Microorganisms

A Laboratory Manual

Roxana B. Hughes • Lee E. Hughes

Third Edition

Microorganisms

A Laboratory Manual

Third Edition

Roxana B. Hughes
Lee E. Hughes
University of North Texas

Kendall Hunt
publishing company

Cover image © Shutterstock, Inc.

Kendall Hunt
publishing company

www.kendallhunt.com
Send all inquiries to:
4050 Westmark Drive
Dubuque, IA 52004-1840

Copyright © 1998 by Debrah E. Beck, Lee E. Hughes, and Gerard A. O'Donovan
Copyright © 2013, 2018 by Roxana B. Hughes and Lee E. Hughes
Revised 2020 printing

ISBN 978-1-7924-2266-9

Kendall Hunt Publishing Company has the exclusive rights to reproduce this work,
to prepare derivative works from this work, to publicly distribute this work,
to publicly perform this work and to publicly display this work.

All rights reserved. No part of this publication may be reproduced,
stored in a retrieval system, or transmitted, in any form or by any
means, electronic, mechanical, photocopying, recording, or otherwise,
without the prior written permission of the copyright owner.

Published in the United States of America

CONTENTS

Preface v
Laboratory Safety vii

SECTION ONE— INTRODUCTORY TECHNIQUES

Laboratory 1—Metric System and Microscopes 3
Laboratory 2—Culture Media 13
Laboratory 3—Inoculation of Media; Aseptic Technique 19
Laboratory 4—Bacteria: Nomenclature, Colony Morphology 31

SECTION TWO—STAINING TECHNIQUES

Laboratory 5—Negative Stain 37
Laboratory 6—Smear Preparation 41
Laboratory 7—Simple Stain 45
Laboratory 8—Gram Stain 49
Laboratory 9—Endospore Stain 55
Laboratory 10—Acid-Fast Stain 59

SECTION THREE—BACTERIAL GROWTH AND CONTROL

Laboratory 11—Bacterial Population Count 67
Laboratory 12—Temperature 79
Laboratory 13—Ultraviolet Radiation 87
Laboratory 14—Oxygen Tolerance 93
Laboratory 15—pH 103
Laboratory 16—Osmolarity 107

SECTION FOUR—ANTIMICROBIALS AND EPIDEMIOLOGY

Laboratory 17—Antiseptics and Disinfectants 113
Laboratory 18—Antibiotic Susceptibility 119
Laboratory 19—Epidemiology 125

SECTION FIVE—SURVEY OF MICROORGANISMS

Laboratory 20—Fungi and Lichens 133
Laboratory 21—Cyanobacteria, Algae, and Protozoa 143

SECTION SIX—ENVIRONMENTAL MICROBIOLOGY

Laboratory 22—Water Analysis Membrane Filtration, Colisure Test, MPN 161
Laboratory 23—Microbial Interrelationships 169

SECTION SEVEN—MICROBIAL GENETICS

Laboratory 24—Transformation 179
Laboratory 25—Genetic Mating: Conjugation 189
Laboratory 26—Bacteriophage 195

SECTION EIGHT—BIOCHEMICAL TESTS FOR IDENTIFICATION

Laboratory 27—IMVC 203
Laboratory 28—Sugar Fermentations 211
Laboratory 29—Motility 213
Laboratory 30—Other Biochemical Tests 217
Laboratory 31—Unknown Identification 223

SECTION NINE—APPENDICES

A—Media and Biochemical Formulations 229
B—References and Suggested Readings 239

PREFACE

This laboratory manual builds upon the foundation created in previous editions. We would like to thank Dr. Gerard A. O'Donovan and Dr. Debrah E. Beck for their help in laying that foundation in the original version of this manual. We also acknowledge the contribution of Dr. Daniel A. Kunz who provided the protocol and strains used in the conjugation experiment.

This edition of the manual has been enhanced through the addition of clear learning objectives for each laboratory experience. We hope this will assist both students and their instructors by providing clear goals and expectations for learning in the laboratory.

The lab portion of the course is designed to enhance the understanding of the lecture material, both lab and lecture materials are synchronized as much as possible.

All of the experiments have been student-tested and modified whenever necessary. The last few weeks of the course are devoted to the identification of unknowns, where the student gets an opportunity to use what was learned during the semester. This has been particularly rewarding.

The goals of the course are to prepare students for upper division microbiology and molecular biology courses both here and elsewhere, for careers in medical and cytotechnology, nursing, and clinical microbiology, for curricula in medical, dental and veterinary school and to provide broad, general microbiology training for those students for whom this is their only microbiology course.

R.B. Hughes

L.E. Hughes

LABORATORY SAFETY

Follow the safety regulations at all times in the Laboratory

Following a few simple guidelines will help create an atmosphere in the laboratory that is both safe and conducive to learning for you and your fellow students. These must be followed carefully. Disregard will endanger your health, as well as that of others in the laboratory.

1. Absolutely no eating, chewing gum, drinking, smoking in the lab
2. Never touch your face, bite your nails, apply cosmetics, or manipulate your contact lenses in the laboratory.
3. Storage of food is prohibited in the microbiology laboratory
4. Do not place anything in your mouth while working in the lab. There are chances that it has come in contact with infectious materials.
5. Always wash your hands before and after completing your laboratory work.
6. Wipe down lab bench with disinfectant before and after each laboratory period to minimize bacterial contamination.
7. Leave hats, coats, book bags, and other items on the coat and book rack at the laboratory entrance. Cluttered walkways create hazardous conditions.
8. Do not "migrate through the lab" with a loop or pipette containing infectious materials.
9. When transferring cultures, always flame needles, loops, and mouths of test tubes or flasks before and after each transfer.
10. Always cool inoculating loops and needles after flaming to avoid sizzling and the creation of aerosols.
11. Always transport tubes in racks to avoid spilling of bacterial cultures. Tubes should always be placed in racks, never laid on table tops.
12. No open toed shoes may be worn to the laboratory, *i.e* no sandals, flip-flops, crocks, slides, etc. If a student is wearing open toed shoes, the student must leave the lab and will get an absence for the day.
13. Wear gloves when there is a rash/cut on the skin, or when instructed by your lab instructor. Gloves must not be worn outside the lab.
14. Wear eye protection, mask or face shield when instructed by your lab instructor.
15. When pipetting, use rubber pipette fillers or safety pipette pumps. Never "mouth pipette"
16. Use only institution-provided marking pens and writing instruments. These remain in the lab at all times, and must be disinfected on a regular basis.
17. **Dispose of Biohazard Materials Properly**

MATERIAL	**HOW TO DISPOSE**
Contaminated Petri plates	Biohazard containers (with orange bags)
Contaminated tubes (broths/slants)	Place tubes upright in test tube racks in designated area. Organize tubes by sizes as instructed.
Contaminated flask/bottles/etc	Place in designated area. Leave caps/lids on.
Contaminated glass pipettes	Tall beakers (with bleach)

Contaminated Broken glass	Broken glass containers (after they have been decontaminated
Contaminated pipette tips	Benchtop biohazard containers
Transfer pipettes	Benchtop biohazard containers
Cotton swabs	Benchtop biozard containers
Needles/razor blades	Sharps containers
Cotton swabs	Benchtop biohazard containers (swabs only)
Non contaminated paper towels	Regular trash cans

Follow instructions given by your laboratory instructor. If you have any question, ask your instructor.

18. **Spills of bacterial cultures**- notify the instructor so that the area can be decontaminated. All bacterial spills must be cleaned using **5%bleach** and **wearing gloves**.
    - Dispose all contaminated material (including gloves) in the autoclave trash. Wash your hands after finishing the clean-up.
19. Notify the instructor immediately if you cut or scratch yourself. Wash the wound with soap and water and employ appropriate first aid (First aid available in the lab)
20. Report any broken glass to your laboratory instructor immediately after the accident happened**.**
21. Turn off the aseptic burners before leaving the laboratory.
22. Only registered microbiology students are allowed in the microbiology lab. **No unauthorized persons in the microbiology lab**.
23. Carry microscopes in the appropriate manner. The cord should be secured, and both hands should firmly grip the microscope, one under the base and the other on the arm. Always store the microscope with the lowest power objective in place and the stage at its lowest position. This will help prevent damage to the lenses. Remove slides from the stage and clean oil from lenses before storing.
24. Turn off all equipment and return supplies to their appropriate storage area at the end of the laboratory.
25. Always ask your instructor if you are not sure of a procedure or how to use equipment. This could prevent damage to equipment or possible danger to yourself.
25. Immune-compromised students (including those who are pregnant) and students living or caring for immunocompromised individuals will need to consult physicians to determine the appropriate level of participation in the laboratory. (https://www.asm.org/biosafev-guidelines)
26. **Do not remove any materials/bacterial culture from the microbiology laboratory.**

SECTION ONE

Introductory Techniques

LABORATORY ONE

Metric System and Microscopes

LEARNING OBJECTIVES

Students will be able to:

- Correctly utilize and convert metric units of measurement
- identify the parts of the compound light microscope and demonstrate proper use of this microscope
- define magnification, resolution and refraction
- describe the different kinds of microscopes used in microbiology
- focus using oil immersion lenses

PURPOSE

To become familiar with common units of metric measurements used in microbiology. To understand the function and proper use of the microscope. To learn the techniques necessary for observing microorganisms microscopically.

INTRODUCTION

The metric system and the microscope are very important tools in biology. This lab is designed to introduce you to the concept of the metric system and to the basics of microscopy and to give you practice in using both of these important tools.

THE METRIC SYSTEM

Background

The metric system is the standard in science for units of measurement. The three base units are the meter for length, the gram for mass, and the liter for volume. There are several prefixes that specify whether fractions or multiples of a unit will be used for a measurement. Table 1.1 contains the prefixes and their designations.

If we were measuring lengths of earthworms, our base unit would be the meter. The most practical fraction of that unit for measuring macro-invertebrates would be the centimeter. If we were aliquoting doses of medicine for injections, our base unit would be the liter, and the most practical fraction of that unit for measuring small volumes would be the milliliter. Metric conversions can be performed by a number of methods, but these all involve the memorization of equivalencies and an example of algebraic conversion follows. NOTE: the metric system is based on units of 10!

TABLE 1.1 Common metric units and their designations.

Prefix	Fractional/Multiple	Decimal	Exponential
kilo- (k)	1,000/1	1000.0	10^3
none = base unit (m, g, L)	1/1	1.0	10^0
centi- (c)	1/100	0.01	10^{-2}
milli- (m)	1/1,000	0.001	10^{-3}
micro- (μ)	1/1,000,000	0.000,001	10^{-6}
nano-(n)	1/1,000,000,000	0.000,000,001	10^{-9}

TABLE 1.2 Common conversion factors in the metric system.

1 km = 1,000 m	1 m = 1/1,000 km
1 m = 1,000 mm	1 mm = 1/1,000 m
1 mm = 1,000 μm	1 μm = 1/1,000 mm
1 μm = 1,000 nm	1 nm = 1/1,000 μm

Example algebraic conversion:

1,200 mm = __________ m

$$(1{,}200mm)\left(\frac{1m}{1{,}000\ mm}\right)=\left(\frac{(1{,}200mm)(1m)}{(1{,}000\ mm)}\right)=1.2m$$

If we know how many mm there are in a meter, then we have a conversion factor to multiply by the original measurement. Always set up the equation so that the original units cancel out. This will leave the desired units in the answer.

Here is a general rule that may help you with your calculations: If you are converting a larger unit to a smaller one (meter to cm) then move the decimal to the right; if you are converting a smaller unit to a larger one (meter to km) then move the decimal to the left.

Microscopes: Microbiologists must view the organisms they work with and this must be done using some form of microscope. A variety of modern light microscopes are used to view bacteria including bright-field, dark-field, phase-contrast and fluorescent microscopes. Structural details of bacterial cells or viruses must be viewed with one of two types of electron microscopes, the transmission electron microscope (TEM) and the scanning electron microscope (SEM).

A modern compound microscope (Fig. 1.1) has three separate lens systems. These are (1) the **condenser** lens situated between the light source and the specimen. Light from a lamp or other source is focused on the specimen by the condenser lens, (2) the **objective** lens is placed next to the specimen and produces a real magnified image of the microscopic field within the dark tube of the microscope and (3) the **ocular** lens (**eyepiece**) enlarges this image further so that it can be perceived as a virtual image by the eye.

Magnification of a compound microscope is determined by multiplying the magnification of the objective lens by that of the eyepiece lens. The latter is usually 10X while the objective lenses, mounted on a rotating nosepiece (Fig. 1.1), give magnifications of 10X (low power), 40X (high, dry power) and 100X (oil immersion). The resulting magnifications are therefore 100X, 400X and 1000X.

The importance of the condenser lens, though not directly involved in image formation, is often overlooked. Correct adjustment of the condenser lens is of critical importance in providing a clear

Blocked due to copyright restrictions.

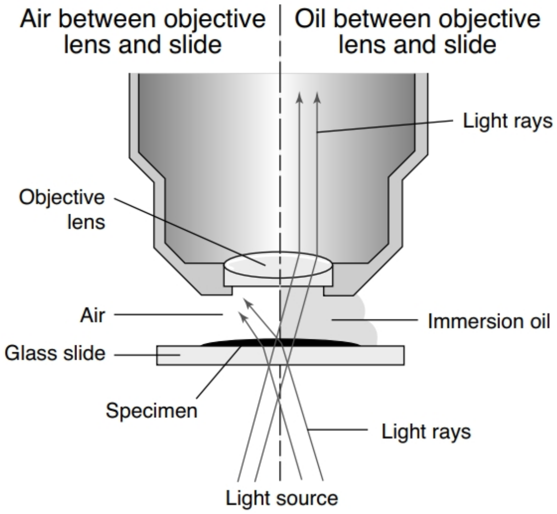
image. At high magnification, the condenser must be positioned to provide critical illumination of the microscopic field. In other words, rays from the light source must be brought to focus in the plane of observation as the field of light transmitted must almost fill the objective lens. Lack of brightness of an object becomes a limiting factor as the specimen is enlarged. The light intensity is adjusted by opening or closing the iris diaphragm located near the condenser (Fig. 1.1).

Resolution: At first glance, one might think that magnification using the compound microscope could be increased indefinitely. This is not the case as the limit is set by the physical properties of light. Because of the wave nature of light, a small object appears as a disc surrounded by a series of light and dark rings. Two adjacent points can be distinguished as separate or **resolved**, only if the rings surrounding them do not overlap. The distance, d, between two points that can be distinguished from one another as two separate entities is the **resolving limit** or **resolution** of the instrument and it is this property that determines the maximal useful magnification of the light microscope.

Bacteria average about 1 μm in diameter so that the compound light microscope is ideal for examining them being able to resolve objects that are within 0.2 μm of each other.

Refraction: The formation of an image by a lens depends on the refraction of light. Refraction results from a change in velocity of light when it passes from one medium to another.

For example, the refractive index of ordinary glass is about 1.5 while that of air is 1.0. Thus, light travels 1.5 times faster in air than in glass. The frequency of light remains constant, but the wave length decreases as the refractive index of the medium increases.



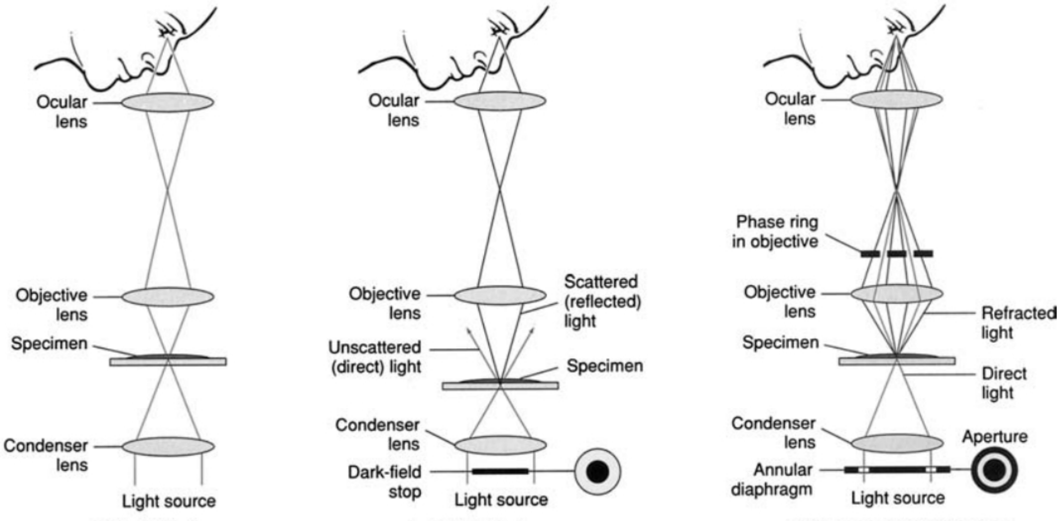
Copyright © Kendall Hunt Publishing Company

Refraction results if light passes an interface between media of different refractive index at some angle to "normal" or perpendicular.

The importance of understanding refraction is clearly seen when one uses a 100X objective. Light from the condenser passes through the specimen (on a glass slide), then through air, and finally into the objective (glass). Since air is less dense than glass, considerable refraction would occur between the specimen and the objective. By using a liquid with a higher refractive index than air to fill in the space between the specimen and the objective, one could obviate this problem. Cedar wood oil ideally satisfies as such a liquid. It has the same refractive index as glass, namely 1.5, and this effectively eliminates refraction between the specimen and the objective.

Other types of microscopy are now summarized.

Bright-field microscopy: As mentioned above, the most commonly used instrument to view microorganisms is the compound bright-field microscope. It has a condenser lens which collimates light onto the specimen, objective lenses near the specimen that magnify it and an ocular lens near the eye that magnifies the image. Light rays pass through the specimen in bright-field microscopy. Since objects are seen against a light background, staining the


Ocular lens
Objective lens
Specimen
Condenser lens
Light source
Ocular lens
Objective lens
Scattered (reflected) light
Unscattered (direct) light
Specimen
Condenser lens
Dark-field stop
Light source
Ocular lens
Phase ring in objective
Objective lens
Refracted light
Specimen
Direct light
Condenser lens
Aperture
Annular diaphragm
Light source

a. Bright-field microscope b. Dark-field microscope c. Phase-contrast microscope

Copyright © Kendall Hunt Publishing Company

specimen enhances its contrast against the background. Specimens that are stained and viewed with the bright-field light microscope appear dark against a brightly lit background.

Dark-field microscopy: Whereas bright-field illumination is used most frequently for observing microorganisms in the laboratory, there are times when dark-field illumination is required for special specimens, where high contrast is required. When light shines on a very small object, some light is scattered making the object appear luminous and visible against a dark background. The technique of dark-field illumination takes advantage of this phenomenon and thereby permits the detection of objects so small as to otherwise provide insufficient contrast. A special kind of condenser is used which focuses a hollow core of light onto the specimen with the diverging rays not entering the objective. To achieve this, an opaque disc or dark-field stop is placed in the center of the substage condenser. This stop blocks direct light in the center of the condenser and does not allow it to reach the specimen. Only those light rays that are deflected by the specimen enter the objective and are observed. The specimen appears light against a dark background. Dark-field microscopy is useful for visualizing bacterial flagella which are too thin to be resolved by bright-field microscopy.

Phase-contrast microscopy: Fritz Zernicke assembled the first phase-contrast microscope in 1932. This microscope has the capacity to detect minute differences in refractive indices of the specimen and its surrounding medium. The low contrast of living cells as viewed with a bright-field microscope can be greatly increased by using an instrument with a modified optical system known as phase-contrast microscopy. This is done by mounting an annular aperture diaphragm below the substage condenser so that the specimen is illuminated with a ring of light. Rays from the light source pass around the diaphragm in the substage condenser and illuminates the specimen with the ring. Phase-contrast microscopy is based on the fact that the rate at which light travels through objects is inversely related to their refractive indices. Since the wave-length of light is independent of the medium through which is travels, the phase of a light ray passing through an object of higher refractive index than the surroundings is somewhat retarded. The system of rings in the condenser and the objective separate the rays diffracted from the specimen from those that are not.

Fluorescence microscopy: The fluorescence microscope is used to identify microorganisms and other specimens that fluoresce. Fluorescence may be a natural property of the specimens due to the presence in the sample of fluorescing compounds, such as chlorophyll, or fluorescent dyes, such as acridine orange, which are added to the sample. The fluorescent microscope is similar mechanically to conventional microscopes but it uses dark-field illumination and shorter wavelengths of light, those in the blue and ultraviolet region of the spectrum. When these short wavelengths strike certain compounds, the sample fluoresces naturally. In other words, the specimen emits light at a longer wavelength in the visible spectrum which can then be visualized using appropriate light filters. Immunofluorescence involves the use of antibodies that are linked to a fluorescent dye. Fluorescent microscopy is used in the study of microbial structures, in medical and food bacteriology to detect pathogens, and in microbial ecology to detect and count bacteria in soils and water.

In the following exercises, you will practice using the microscope to observe microorganisms.

LABORATORY EXERCISE

1.1 Wet Mount Technique.

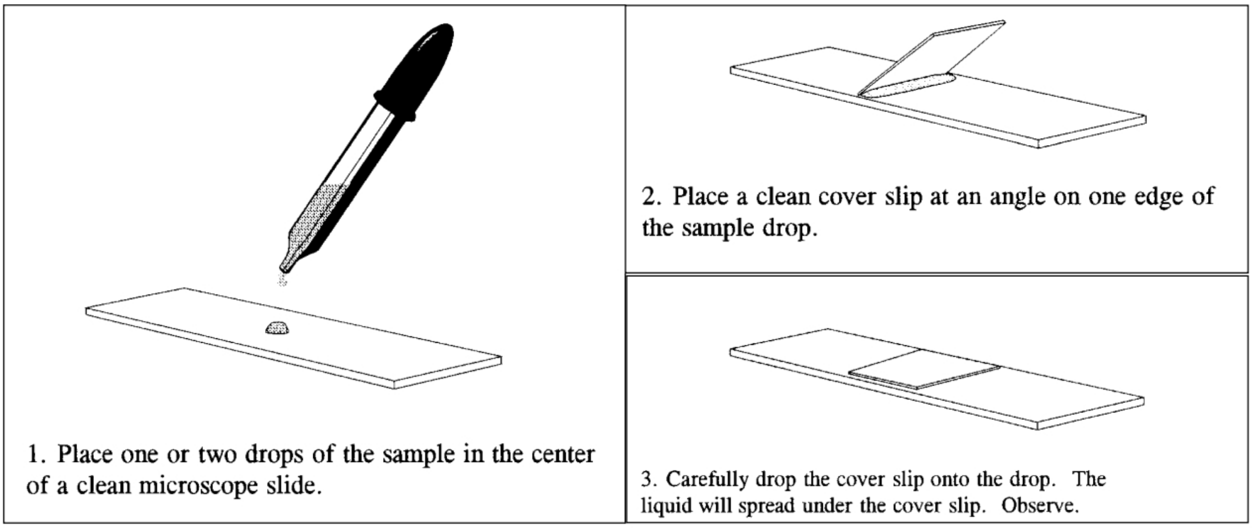
MATERIALS

- ☑ Hay infusion
- ☑ Pond water
- ☑ slides and cover slips
- ☑ Pasteur pipettes or transfer pipettes and bulbs

PROCEDURE

Each student should prepare 3 wet mounts (Fig. 1.2) from provided hay infusion and/or pond water. Observe wet mounts at low magnification (10X and 40X objectives, see Exercise 1.2) and attempt to identify 10–15 microorganisms (protozoa, algae, bacteria, fungus microscopic invertebrates) using lab manual and TA as sources.

FIGURE 1.2 Wet mount



1. Place one or two drops of the sample in the center of a clean microscope slide.

2. Place a clean cover slip at an angle on one edge of the sample drop.

3. Carefully drop the cover slip onto the drop. The liquid will spread under the cover slip. Observe.

1.2 Microscopic Observations: High-dry Objectives.

PROCEDURE

1. Lower the stage. Place slide containing specimen onto the stage.
2. Observe with low power objective (10X). Focus using course adjustment, then fine adjustment.
3. If further magnification is desired, rotate the revolving nosepiece to bring the next high-dry objective (40X) into position. A parfocal microscope should require only minor focusing with the fine adjustment. To prevent damage to the lens, never use the course adjustment with any objective lens other than the 10X or lower.
4. When observations are complete, remove slides and store the microscope in the appropriate place with the lowest power objective in place (generally 10X) and the stage in its lowest position to prevent possible damage to the lens.

1.3 Microscopic Observations: Oil-immersion Objectives

PROCEDURE

1. Focus the specimen as described in Exercise 1.2 using the 40X objective. *It is very important to carefully focus before continuing this procedure.*
2. Rotate the revolving nosepiece to between the 40X and 100X objectives. Add a small drop of immersion oil to the area of the slide under observation.
3. Rotate the revolving nosepiece to position the 100X objective over the specimen. The lens should touch the oil droplet.
4. Observe. If the specimen is not visible, carefully turn the fine adjustment in small increments until the sample is in focus.
5. When finished, carefully clean the lens with lens paper and lens cleaner to remove any oil. *Take care not to get immersion oil on any of the high-dry lenses. If this occurs, they must be cleaned immediately to prevent damage to the lens.* Store microscope as described in previous exercise.

LABORATORY REPORT 1
MICROSCOPES

Name:______________________________________

RESULTS

Observe and draw below 10–15 organisms from the pond water or hay infusion. Identify as many of these as you can. (use illustrations in Laboratories 20 and 21 lab manual for reference)

QUESTIONS

1. Name the type of microscope you are using: ____ .

2. The maximum useful magnification achievable with these microscopes is _________X.

3. The microscope should be stored with the ________X objective and the stage in its ____________ position to prevent damage to the lenses.

4. ______________________ is the term which describes the ability to change objectives without having to refocus the microscope.

5. Why is immersion oil needed when using 100X objective?

6. What is the resolution of the brightfield microscope?

7. What is the function of the diaphragm?

Briefly define or give the purpose of the following:

8. Resolution—

9. Magnification—(calculate using a 5X ocular and a 20X objective. Show your work.)

10. Light source—

11. Course focus adjustment—

12. Refractive index

13. Wet mount—(briefly diagram procedure)

14. Condenser—

Metric calculations practice

1. 345nm = ____________________um
2. 2nm = ______________________m
3. 1ng = _______________________g
4. 250μl = _____________________ml
5. 3ml = _______________________μl
6. 35g = _______________________mg
7. 1mg = _______________________g
8. 1cm = _______________________mm
9. 1000ul = _______________________ml
10. 1000ml = _______________________L

LABORATORY TWO

Culture Media

LEARNING OBJECTIVES

Students will be able to:

- describe how to prepare culture media
- categorize different types of culture media based on type of use and by chemical composition

PURPOSE

To learn the principles of microbial nutrition.

INTRODUCTION

Nutrients are the substances that all organisms require as a source of energy and for synthesis of cell materials. For many different organisms, including bacteria, organic compounds are used as a source of carbon and energy. However, all bacteria are not restricted to the use of carbohydrates as the sole source of carbon and energy. Photosynthetic organisms, including photosynthetic bacteria, utilize CO_2 as the sole source of carbon, but conversion of carbon dioxide to carbohydrates is a very expensive reaction for the cell, using many ATP molecules.

Bacteria are classified in two groups based on their requirement for carbon. Heterotrophs obtain their carbon from organic compounds and autotrophs obtain their carbon form carbon dioxide.

The energy source can come from organic molecules or though photosynthesis.

Within a cell, whether bacterial or eukaryotic, the molecular components are similar. While small molecules such as carbon (C), hydrogen (H_2), oxygen (O_2) and nitrogen (N) are taken in from the environment, macromolecules, such as proteins and nucleic acids (DNA and RNA) are synthesized inside the cell. In fact, the cell mass is made primarily of C, H, O and N. However, other elements are needed in smaller amounts or even in trace quantities for the proper functioning of enzymes and nucleic acids.

The minor minerals required are phosphorus, potassium, iodine, sulfur, calcium, iron, magnesium, zinc, manganese, copper, molybdenum and cobalt.

Another important molecule that aids the metabolism of nutrients is water. A bacterial cell is 70–90% water.

For strict aerobes and facultative anaerobes, oxygen is another element used by the cell for oxidation of various molecules.

A culture medium is the substance containing all the necessary nutrients for growth of an organism. There are thousands of different growth media because what one bacterium grows on does not necessarily maintain the growth of another. Therefore, the proper nutrient balance must be maintained for each bacterium. There are general growth media, such as tryptic soy broth and nutrient agar. These two types of growth media are considered **"rich", complex** or **undefined** because the exact components added to such media cannot be determined. Some of the ingredients of nutrient broth include beef extract and peptones.

A second type of growth medium is a **defined, chemical** or **synthetic medium**. An example of defined medium is *E. coli* minimal medium in which the exact chemical makeup is known:

10.5 g K_2HPO_4

4.5 g KH_2PO_4

1.0 g $(NH_4)_2SO_4$

0.5 g Na_3citrate

1000 ml H_2O

Nutrient broth is an example of a rich medium. Its ingredients include:

beef extract – 3 g
Peptones – 5 g
1000 ml H_2O

Isolation of pure cultures of organisms cannot be accomplished by growing organisms in liquid broth, therefore a gelling agent must be added. Many organisms can liquefy gelatin and it melts at room temperature, therefore it is not possible to use that compound for solidifying broth. Agar, a compound from seaweed, is used in modern microbiology and is ideal for the purpose of making agar plates. It is degraded only by a few organisms and remains solid at temperatures near 100°C.

Some organisms require **growth factors** that are specific organic compounds (e.g. vitamins), which cannot be synthesized *de novo* and therefore must be furnished in the medium. Some examples of growth factors are biotin (needed for fatty acid biosynthesis; cobalamin (reduces and transfers single carbon fragments and synthesis of DNA; thiamin (decarboxylations; vitamin K group (electron transport) and others.

In this laboratory exercise three different preparations of growth media are made:

1. nutrient agar slants: used to maintain stock cultures;
2. nutrient agar plates: used to isolate pure cultures of organisms and to do plate counts; and
3. nutrient broth tubes: used to grow large numbers of organisms.

After preparation of the different media, all must be sterilized in an autoclave at 15 pounds per square inch (p.s.i.) steam pressure, at 121°C for 15 minutes. After proper cooling, the different media may be inoculated with bacterial cultures.

LABORATORY EXERCISE

2.1 Culture Media Preparation.

MATERIALS

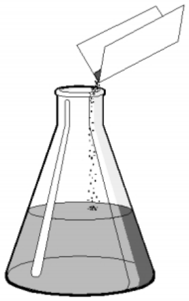
- ☑ Nutrient broth powder
- ☑ Agar
- ☑ Ehrlenmeyer flask (1L)
- ☑ graduated cylinder
- ☑ 10 culture tubes with slip tops
- ☑ 10 culture tubes with screw caps
- ☑ 10–12 Petri plates
- ☑ balance
- ☑ hotplate with stirrers
- ☑ aluminum foil
- ☑ magnetic stir bar
- ☑ pipette helpers
- ☑ 5ml pipettes
- ☑ hot handle holder

PROCEDURE

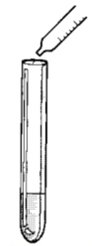
Each lab group will prepare broth tubes, slants and plates.

1. First, dissolve 2.8 g of Nutrient Broth powder in 350 ml dH_2O using the flask containing a stir bar.
2. Dispense 5 ml each into 10 slip top tubes for a total of 50 ml. Cap and set aside. These are the Nutrient Broth tubes.
3. Add 5 g of agar to the remaining 300 ml of liquid.
4. Using medium heat to dissolve the agar while stirring constantly. Watch carefully. Remove from heat when agar has dissolved, and liquid is completely clear.
5. Dispense 5 ml each into 10 screw cap tubes for a total of 50 ml. Cap loosely and set aside. These will be the Nutrient Agar slants.
6. Cover the flask which contains the remaining 250 ml of Nutrient Agar with foil. Place the flask and racks containing the broth and slant tubes into an autoclave. Set such that the contents will be at **121°C at 15 p.s.i. for a minimum of 15 minutes**.
7. Remove all items from the autoclave. Place the slant tubes at an angle (a 2 inch stack of paper towels placed under the corner of the rack will generally give the correct angle) and allow to cool. Broth tubes can be set aside for later use.
8. Cool the flask contents to 50–60°C. Using a burner, flame the mouth of the flask and then pour about 25 ml of the agar mixture into each of ten sterile petri plates (pouring until the bottom of the plate is just covered will give appropriate amount). Immediately cover each plate and allow to cool while resting on a flat surface. Once cooled, store all the prepared media in a cool, dry place until use in the next laboratory experiment. Make sure to work close to the Bunsen burner to prevent contamination of the plates.
9. During next class period, observe media and assess sterility and proper preparation.

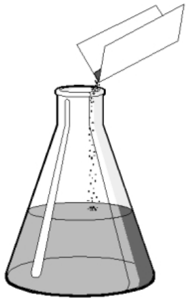
FIGURE 2.1 Culture media preparation procedure.



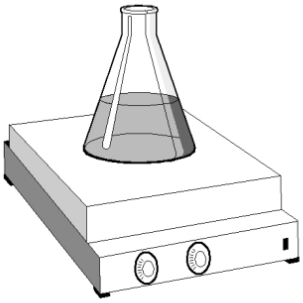
1. Dissolve 2.8 g of Nutrient broth powder in 350 ml of water in flask containing a stir bar.



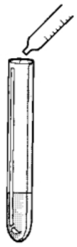
2. Dispense 5 ml each into ten slip top tubes for a total of 50 ml. Cap and set aside these Nutrient broth tubes.



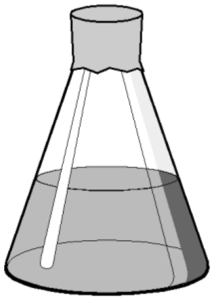
3. Add 5 g of agar to the remaining 300 ml of liquid.



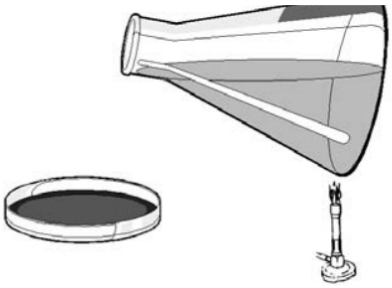
4. Heat to dissolve the agar while stirring constantly. Watch carefully and remove from heat before it boils.



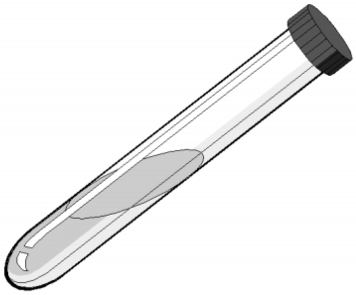
5. Dispense 5 ml each into ten screw cap tubes. Cap loosely and set aside as Nutrient agar slants.



6. Cover flask with foil. Autoclave flask, Nutrient broth tubes, and Nutrient agar slants.



7. Cool flask contents to 50–60°C. Aseptically pour into ten sterile Petri dishes. Cool plates on flat surface.



8. Place slant tubes at an angle and cool. Broth tubes can be set aside for later use.

LABORATORY REPORT 2
CULTURE MEDIA

Name:______________________________________

RESULTS

2.1 Culture Media Preparation

Indicate successful completion of the media preparation by obtaining the initials of your teaching assistant for each type of media:

________ Broth tubes ________ Slants ________ Plates

2.2 Observation of Prepared Media

- Did any of the plates get contaminated? If so, where do you think the contamination is coming from.

- Did all the nutrient agar slants solidify?

- Were you successful in preparing all of the types of media in this laboratory? If not, describe the problem and identify possible reasons it occurred.

QUESTIONS

1. The ingredient added to make solid or semi-solid media is _____________.

2. The compound in Ques. #1 will not go into solution until the media have been _____________________.

3. Identify the differences between:

Synthetic medium: ____

Complex medium:____

4. Name three constituents or ingredients (general) of media which are required for bacterial growth:

5. Name the proper autoclaving conditions

   a) Temperature
   b) Time
   c) Pressure

6. At what temperature does agar solidify after it has been dissolved?

7. What is the agar concentration in solid media?

LABORATORY THREE

Inoculation of Media; Aseptic Technique

LEARNING OBJECTIVES

Students will be able to:

- isolate pure cultures
- use aseptic technique
- describe the use and applications of selective and differential media

PURPOSE

To learn proper aseptic technique and practice the skills necessary for isolation and propagation of bacterial cultures.

To learn about Selective and Differential Media.

INTRODUCTION

Bacteria in nature are generally present in mixed cultures. For study in the laboratory, these bacteria are separated and cultured as pure cultures. A **pure culture** is one which contains only one type of bacteria. Since each colony on a plate arises from a single bacterial cell, a pure culture can be obtained from an isolated colony on a plate which contains only one colony type (*i.e.* is not contaminated with other organisms). Pure culture technique allows the microbiologist to examine the unique biochemical, morphological, and physiological properties of each bacterium.

In order to work with bacteria in the laboratory, it is necessary to utilize proper aseptic technique. **Aseptic technique** is a combination of laboratory methods which are used to keep the worker and work area free of bacterial contamination, as well as insure that culture media are inoculated only with the organism of interest. Practice of good aseptic technique includes cleaning of the work area with a disinfectant both before and after use. Always work near a flame, keep all sterile containers closed except during use, never lay caps on the bench, keep all tubes in racks and perform all procedures in a quick, orderly fashion, and work in a clutter free area. Each exercise in this chapter details the appropriate methods for inoculation of the different kinds of media using aseptic technique.

In this exercise, you will learn the techniques for isolation and growth of pure cultures. This includes the streak plate technique for isolation of colonies, inoculation of broths for propagation of cultures, and inoculation of slants for storage of cultures. Other culture techniques are described elsewhere in this manual.

Selective and Differential Media

Selective media are used to isolate particular groups of organisms while suppresing the grow of others. Some substances used to suppress growth are antibiotics, sodium chloride and certain dyes among others.

Differential media allow the differentiation of colonies of desired organisms from others that grow in the same plate.

Enriched Media

Blood agar plates contain 5% of sheep blood which allows fastidious organisms to grow. In addition, it is a differential whose main role is the visualization of different hemolytic patterns.

LABORATORY EXERCISE

ORGANISMS

(liquid culture in tube)

☑ *Bacillus megaterium*

☑ *Escherichia coli*

☑ *Pseudomonas fluorescens/P. putida*

☑ *Staphylococcus saprophyticus*

☑ *Staphylococcus epidermidis*

☑ Mixed Culture (*E. coli, Micrococcus luteus, Serratia marcescens*)

3.1 Inoculation of Broths.

MATERIALS

☑ Inoculating loop

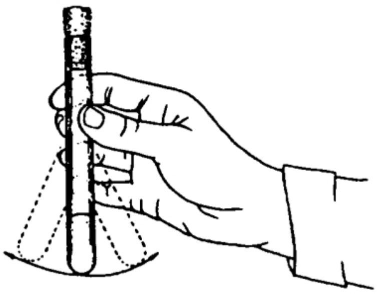
☑ 1 Nutrient broth tube

PROCEDURE

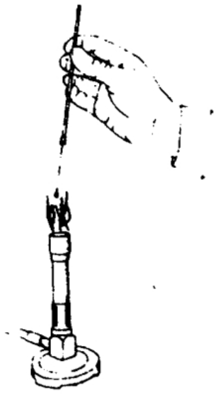
Each student selects one of the first five organisms listed above and inoculates a broth tube with that organism.

1. Sterilize the inoculating loop by placing the metal wire in a burner flame until red hot. Remove from the flame and cool 15–20 seconds.
2. Remove the lid from the culture tube and grip with the last two fingers of the hand holding the loop. Pass the mouth of the tube through the flame.
3. Dip the inoculating loop into the culture and remove a sample. Again flame the mouth of the tube, replace the cap, and return to the tube rack.
4. Open the broth tube to be inoculated and flame the mouth. Insert the inoculating loop with culture and gently swirl in the liquid. Remove the loop, flame the mouth of the tube, and replace the cap.
5. Flame the loop to remove any residual culture. The inoculated tube may now be incubated at the appropriate temperature.

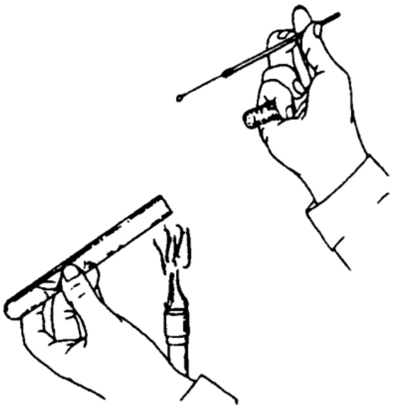
FIGURE 3.1 Inoculation of broth tubes.



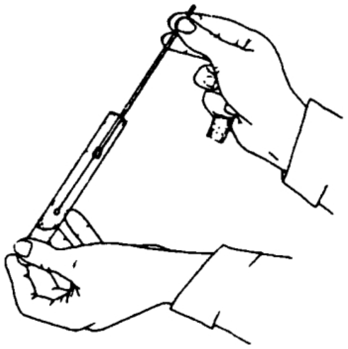
1 Carefully Shake the tube from side to side to put the organisms in suspension. Keep tubes upright to avoid cultures reaching the cap and causing a spill.



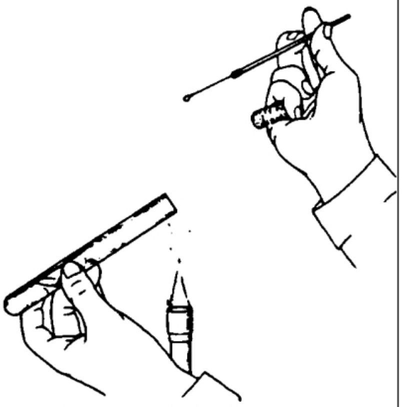
2 Flame the entire loop and wire until it becomes red-hot. Flame the handle slightly also.



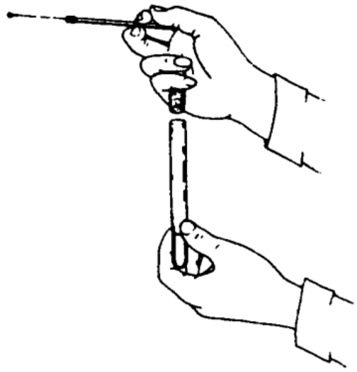
3 Remove the slip top cap and flame the neck of the tube. Don't contaminate the slip top cap by placing it on the table.



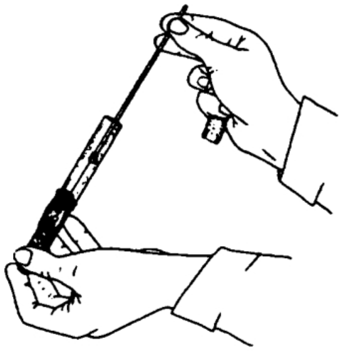
4 Remove a loop full of organisms after the loop has cooled for at least five seconds. Do not touch the sides of the tube.



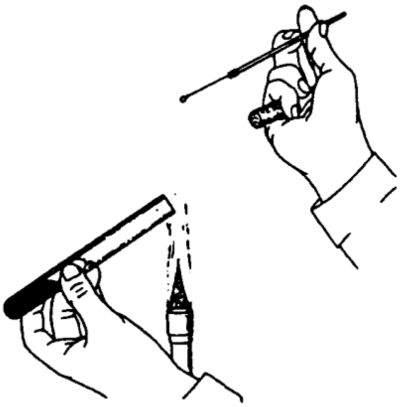
5 Flame the tube again.



6 Return the slip top cap to the tube. Place the tube in a rack.



7 Inoculate broth.



8 Flame mouth of tube.

3.2 Inoculation of Slants.

SUPPLIES

☑ Inoculating loop

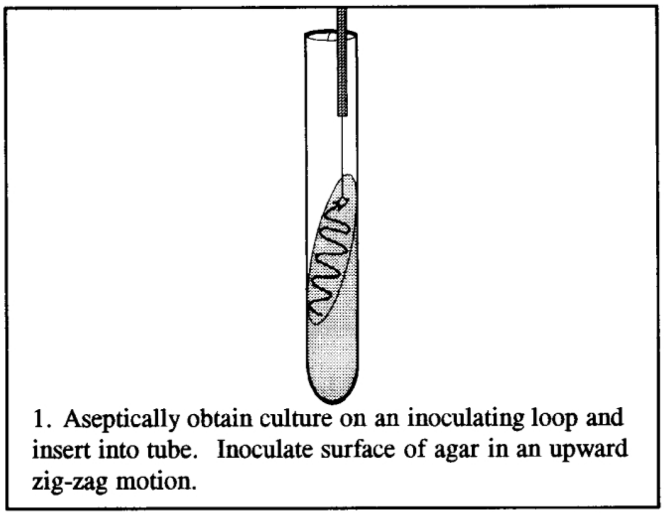
☑ 1 Nutrient Agar slant

PROCEDURE

Each student inoculates the 1 slant with the organism chosen for Exercise 3.1.

1. Obtain a sample of culture on the loop using the method described in Exercise 3.1.
2. Open the slant tube to be inoculated and flame the mouth. Insert the inoculating loop with culture and, beginning at the base of the slant, inoculate the surface of the agar in an upward zig-zag motion (Fig. 3.2). Remove the loop, flame the mouth of the tube, and replace the cap.
3. Flame the loop to remove any residual culture. The inoculated slant may now be incubated at the appropriate temperature.

FIGURE 3.2 Slant inoculation.



1. Aseptically obtain culture on an inoculating loop and insert into tube. Inoculate surface of agar in an upward zig-zag motion.

3.3 Isolation of Colonies—Four Quadrant Method.

SUPPLIES

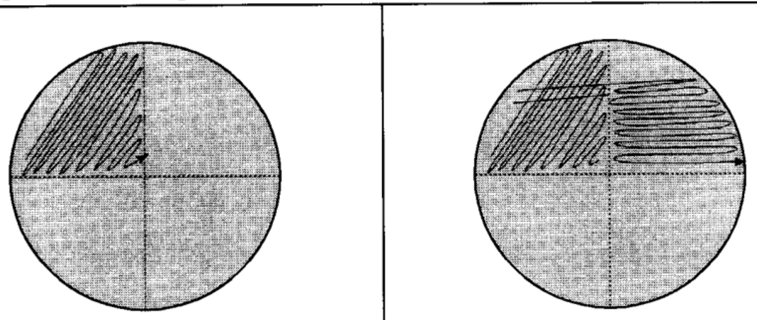
☑ 2 Nutrient Agar plates

PROCEDURE

Each student inoculates 1 Nutrient Agar plates with the organism chosen for Exercise 3.1 and an additional Nutrient Agar plate with the Mixed Culture per table of students.

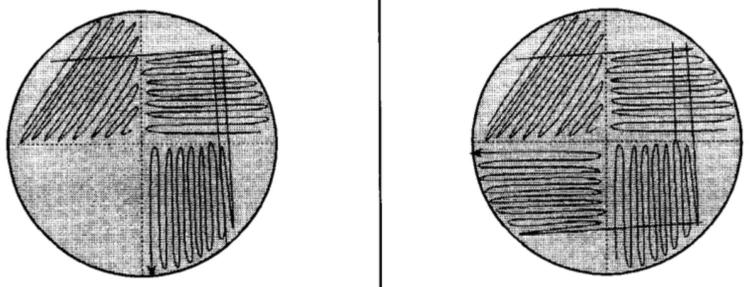
1. Obtain a sample of culture on the loop using the method described in Exercise 3.1.
2. Working near the flame, invert the plate to be inoculated and remove the section containing the agar. Place the tip of the loop gently against the surface of the agar and drag across the surface in a side-to-side motion until one-quarter of the plate has been completely streaked (Fig. 3.3 and Fig. 3.4). Replace the plate on the lid.
3. Flame the loop and cool. Open the plate. Beginning on one side of the first quadrant, drag the loop through the center of the first series of streaks twice and repeat the streaking pattern in the second quadrant of the plate. Be careful not to reenter the first quadrant or touch any of the previous streaks. Replace the plate on the lid.
4. Repeat the streaking pattern for the remaining two quadrants of the plate, flaming and cooling the loop between each. Keep the plate closed while waiting between streaks to minimize the chance of contamination.
5. Flame the loop to remove any residual culture. The inoculated plate may now be incubated in the inverted position at the appropriate temperature. Inverting the plate helps to prevent contamination and limits condensation of water on the agar surface.

FIGURE 3.3 Four quadrant streak pattern.



1. Divide the plate into quadrants. Aseptically obtain culture on loop and make the first streak as shown.

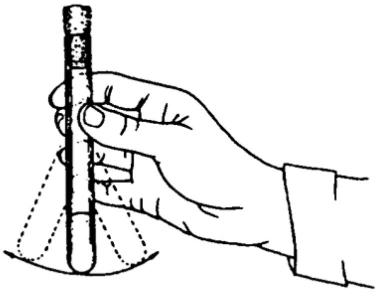
2. Flame loop and cool. Pass loop through first streak and then streak second quadrant as shown.



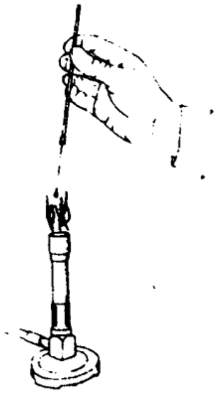
3. Flame loop and cool. Pass through second streak and then streak third quadrant as shown.

4. Flame loop and cool. Repeat streak pattern to fourth quadrant and then flame loop. Incubate plate.

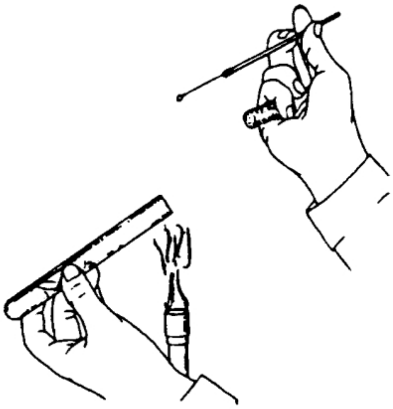
FIGURE 3.4 Inoculation of streak plate.



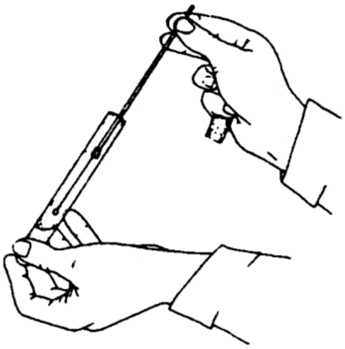
1 Carefully shake the tube from side to side to put the organisms in suspension. Keep tubes upright to avoid liquid reaching the cap and causing a spill.



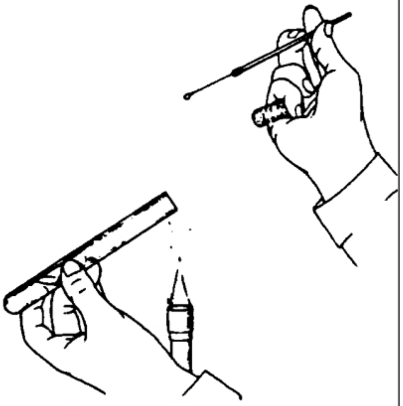
2 Flame the entire loop and wire until it becomes red-hot. Flame the handle slightly also.



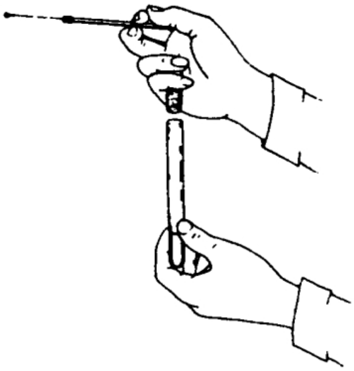
3 Remove the slip top cap and flame the neck of the tube. Don't contaminate the slip top cap by placing it on the table.



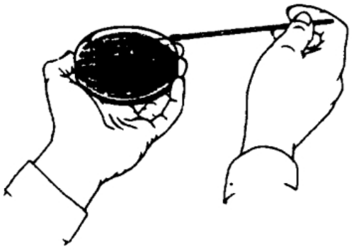
4 Remove a loop full of organisms after the loop has cooled for at least five seconds. Do not touch the sides of the tube.



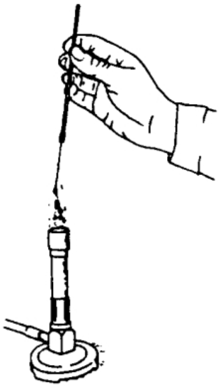
5 Flame the tube again.



6 Return the slip top cap to the tube. Place the tube in a rack.



7 IStreak the plate, holding as shown. Do not gauge into the medium with the loop.



8 Flame the loop before placing it down.

3.4 Selective, Differential, and Enriched Media.

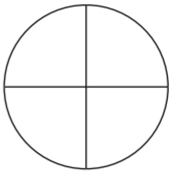
PROCEDURE

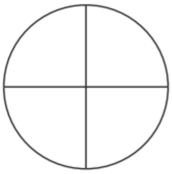
Each table of students will inoculate one of each of the following media: MacConkey, Colistin Nalidixic acid (CNA), Mannitol salt Agar (MSA), and two plates of Blood Agar.

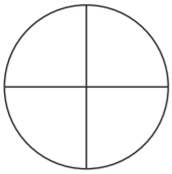
1. Obtain a sample of culture on the loop using the method described in Exercise 3.1.
2. Label the bottom of the plate by dividing it into 3 parts. Label each section with the desired organism. See chart below for organisms on each plate.
3. Working near the flame, obtain a loopful of the organism, invert the plate to be inoculated, and remove the lid. Place the tip of the loop gently against the surface of the agar and drag across the surface in a zig-zag pattern in the assigned section. Replace the plate of the lid.
4. Repeat the streaking patter for all the assigned organisms.
5. Incubate the inoculated plate in the inverted position at the appropriate temperature.

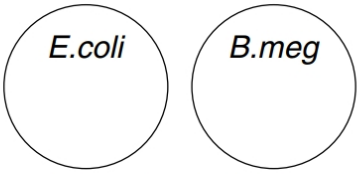
MATERIALS

- ☑ 1 MacConkey agar plate
- ☑ 1 mannitol salt agar (MSA) agar plate
- ☑ 1 colistin-nalidixic acid (CNA) agar plate
- ☑ 2 blood agar plate









Streak for isolation

MacConkey Agar **Selective, Differential**	**Colistin Nalidixic Acid Agar** **Selective**	**Mannitol Salt Agar** **Selective, Differential**	**Blood Agar** **Enriched, Differential**
E. coli	*E. coli*	*E. coli*	*Escherichia coli*
Pseudomonas fluorescens/P. putida	*P.fluorescens/P. putida*	*P. fluorescens/P. putida*	*Bacillus megaterium*
Staphylococcus epidermidis	*S. epidermidis*	*Staphylococcus epidermidis*	
Staphylococcus saprophyticus	*S.saprophyticus*	*S.saprophyticus*	

Rich, Selective and Differential media

Media name	Organisms	Inhibitor	Indicator	Results
CNA Agar (Colistin/Nalidixic Acid agar)	**Growth of Gram-positive organisms (selective for G+)** Suppression of Gram-negative organisms growth	**Colistin, nalidixic acid**		**Growth of Gram-positive** organisms
MacConkey Agar	**Growth of Gram-negative organisms.** Inhibit the growth of Gram-positive organisms.	**Bile salts (inhibit G+)**	**Neutral Red** (pH drop around colony causes the color change)	**Lactose fermenting Gram-negative** appear **pink to brick red** colonies, with or without a zone of precipitation. **Lactose non-fermenters** appear clear.
Mannitol Salt Agar	**Growth of *Staphylococcus* spp**	**Sodium chloride 7.5%**	**Phenol red**	***S.saprophyticus*** produces white/cream colonies and turns the media yellow. (ferments mannitol) ***S.epidermidis*** growns but does not ferments mannitol
Tryptic Soy Agar	**Growth of most Gram-positive and Gram-negative** organisms (used in 2042 labs	**No inhibitor** Mix of beef extracts, peptones, soy meal, other.	**No indicator**	**Growth of the majority of organisms used in 2042 labs.**
Blood Agar Plate	**Growth of most Gram-positive and Gram-negative** Organisms (used in 2042 labs)	**No inhibitor**	**Blood (of hemolysis)**	**Hemolytic patterns** Beta hemolysis Alpha hemolysis gamma hemolysis (Non-hemolytic)

LABORATORY REPORT 3
INOCULATION OF MEDIA; ASEPTIC TECHNIQUE

Name:______________________________________

RESULTS

3.1 Inoculation of Broths

Describe the appearance of the growth in the tubes for each of the organisms inoculated at your lab table:
____

____

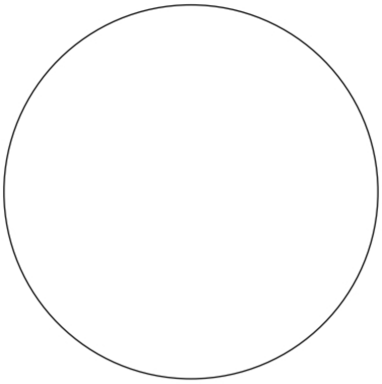
3.2 Inoculation of Slants

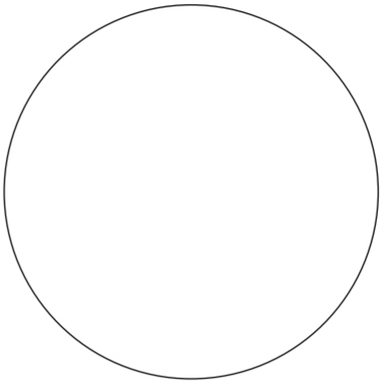
Describe the appearance of the growth on the slants for each of the organisms inoculated at your lab table:
____

____

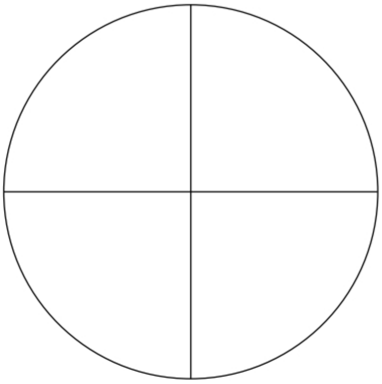
3.3 Isolation of Colonies

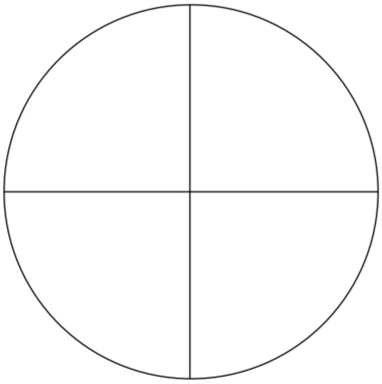
1. Count the number of isolated colonies on your best isolation plate: __________.
2. What are the colors of the isolated colonies on the mixed culture isolation plate?
____
3. Sketch below the growth on each of the plates you streaked for isolation:

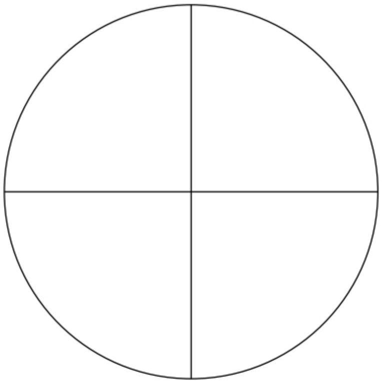


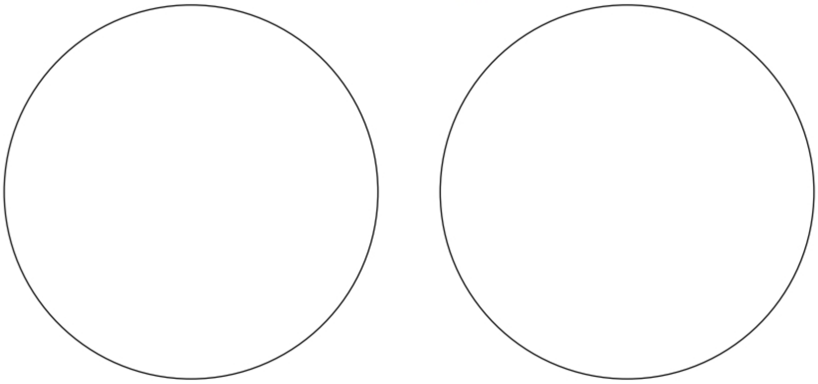


4. Describe the growth pattern for the selective and differential media.


MSA


McConkey


CNA


Blood Agar plates

QUESTIONS

1. Plates should be incubated in the ____________________ position.

2. The loop is flamed in order to make it _____________________.

3. ______ Tubes should be flamed: a) just after opening; b) prior to closing; c) both just after opening and prior to closing; d) never.

True or False 4. It is okay to remove a lid from a tube and place it on the counter while inoculating the tube.

True or False 5. When inoculating, you should always finish with, flame and close the first tube you are working with before opening the next.

6. The purpose of the streak plate technique is to obtain _____________________ colonies.

7. Slants are used for the ____________________ of cultures.

8. _____________________ tubes are used for propagation of cultures.

9. Define a pure culture: ____

____

10. What is the purpose of aseptic technique? ____

____

____

11. Define the following types of media

    Selective ____

    Differential ____

12. MacConkey agar is a medium that is selective for _____________________ bacteria and differential based on _____________________ fermentation.

13. Colistin nalidixic acid is a medium selective for _____________________ bacteria.

14. What is the purpose of using blood agar plates?

Bacteria: Nomenclature, Colony Morphology

LEARNING OBJECTIVES

Students will be able to:

- differentiate colony morphology types
- correctly use binomial nomenclature

PURPOSE

To learn the proper method of naming bacteria using the binomial system of nomenclature; to observe common colony and cell morphology.

INTRODUCTION

Nomenclature is the science of naming organisms. Bacteria are named using the binomial system of nomenclature, as are most organisms. Each bacterium is given two names (binomial), a genus name and a species name which are written in Latin and underlined or italicized. The genus portion is always capitalized while the species is written in lower case. The genus may be abbreviated using only the first letter after writing the entire name one time.

> Example: *Escherichia coli* are Gram negative organisms. Within the large intestine, *E. coli* has found a warm, moist, nutrient-rich home.

Most bacteria have been assigned names for their Domain, Kingdom, Phylum, Class, Order, Family, Genus and Species. The three Domains are eubacteria or **bacteria**, archaebacteria or **archaea** and eukaryotes or **eukarya**. The protozoa, fungi and algae are grouped together in the Eukarya domain.

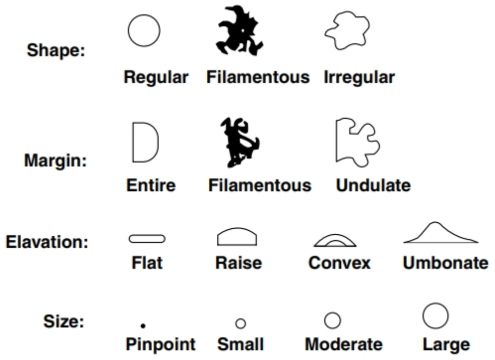
The following morphological characteristics may be taken into account when observing colonies growing on agar plates:

1. Size. The size of different bacterial colonies varies from pinpoint colonies of the lactic acid bacteria (aerotolerant to microaerophilic) to large, spreading colonies as seen for same strains of *Pseudomonas*, *Proteus* or *Bacillus*.
2. Margin or edge. Different bacterial types grow in different colony shapes around the periphery of the colony. There are smooth edges, rounded projections, irregular notches, filamentous, wavy or undulate, lobate, irregular (erose), ciliate, branching, wooly, thread-like, “hair-lock”-like.

3. Elevation. Different characteristics include flat, raised, convex, drop-like, umbonate, hilly, ingrown in the medium, crateriform.
4. Pigmentation. Some bacteria produce pigments which include red (prodigiosin), yellow, brown, blue to green (pyocyanin), and violet.
5. Optical features. The cell may be shiny, opaque, translucent, opalescent or have a green, metallic sheen.

Important observable characteristics that are useful in describing colonies include the following:

FIGURE 4.1 Bacterial colony characteristics.


Shape:
Regular
Filamentous
Irregular
Margin:
Entire
Filamentous
Undulate
Elavation:
Flat
Raise
Convex
Umbonate
Size:
Pinpoint
Small
Moderate
Large

From Fundamentals of Microbiology for Allied Health by Kathleen Dannelly, Angela K. Chamberlain, and William M. Chamberlain. Copyright © 2009 by Kendall Hunt Publishing Company. Used with permission.

In this lab exercise we will observe differences in cell and colony morphology.

LABORATORY EXERCISE

4.1 Examination of Morphology.

ORGANISMS

(isolated colonies on agar plates)

☑ *Bacillus megaterium*

☑ *Escherichia coli*

☑ *Pseudomonas fluorescens/ P. putida*

☑ *Staphylococcus* spp

☑ *Micrococcus luteus*

☑ *Bacillus* spp

MATERIALS

☑ Dissecting microscope (optional)

PROCEDURE

Observe the growth of the bacteria on the plates provided (or from student plates following Exercise 3). Note differences in shape, appearance, and color. Record your observations on the laboratory report.

LABORATORY REPORT 4
BACTERIA: NOMENCLATURE, COLONY MORPHOLOGY

Name:______________________________________

RESULTS

Describe and/or sketch below your observations of the growth of each of the provided organisms on a general medium (use provided samples or student isolation plates from Exercise 3). Include color, size, colony shape and elevation in your description:

Bacillus megaterium

Escherichia coli

Pseudomonas fluorescens/P. putida

Staphylococcus spp.

Micrococcus luteus

Bacillus spp

SECTION TWO

Staining Techniques

INTRODUCTION

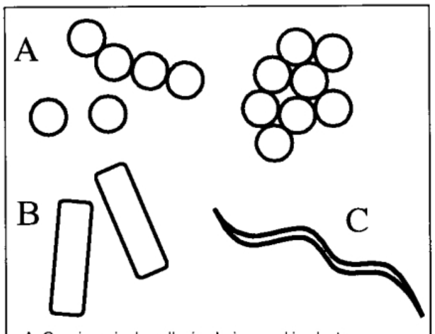
Direct observation of bacteria is difficult with bright-field microscopy due to the near-transparent nature of the bacterial cells. The use of dyes can greatly increase the visibility of these cells, though the cells are killed in the process. In addition, chemical and structural differences between bacterial types can be utilized to differentially stain the cells and helps in the identification of bacteria.

In the following laboratory exercises, several staining methods are given. Each provides a special property which is useful in bacteriological observations of differentiations. Students will practice these methods and learn to identify the results for each bacterial type used in the techniques.

CELL MORPHOLOGY

The morphology of the bacterial cell takes several different forms and sizes. The most common types of bacterial cells to be observed in this laboratory are the coccus (round) such as *Staphylococcus aureus* and *Streptococcus pneumoniae*. There are rod or bacillus-shaped organisms such as *Bacillus subtilis* and *Escherichia coli*. A third morphological type is the spiral-shaped organism, or spirilla, found within the Spirochete family (*Borrelia burgdorferi and Treponema pallidum*). A fourth morphological type is amorphous or having no particular shape, such as *Rhizobium loti* or *Azotobacter vinelandii*. Many bacteria retain one single shape throughout their entire life cycle, but others, such as *Arthrobacter globiformis* and *Brevibacterium linens* change morphology, from rods to cocci, within their life cycles.

Examples of bacterial morphology



A. Cocci as single cells, in chains, and in clusters.
B. Bacilli (rods). C. Spirillum.

Some bacteria are arranged in clusters of cells that resemble grapes (*Straphylococcus aureus*) while other bacteria form chains (*Bacillus* and *Streptococcus*) and still others remain separate cells.

LABORATORY FIVE

Negative Stain

LEARNING OBJECTIVES

Students will be able to:

- successfully prepared a negative stain
- identify and distinguish encapsulated organisms

PURPOSE

The negative stain is usefull for examination of bacterial cell morphology under oil-immersion.

INTRODUCTION

The negative stain provides a relatively simple and effective method for observing bacterial cell morphology. The negative stain is prepared by using a solution of **nigrosin** stain which is mixed with the bacteria and then spread across the surface of a slide. The negatively charged stain does not react with the bacterial surface, which is also negatively charged, so that the film dries to form a dark background through which the bacteria are visible. Since no heat is applied to the slide, there is little change in cell size, although bacteria can sometimes appear larger than life due to the presence of a polysaccharide capsule (see below). In most cases, cell size can be accurately measured using this staining method. The negative stain is also useful for observing cell shape or associations between cells (*i.e.* chains, tetrads, *etc*.).

Some bacteria are capable of producing extracellular **capsules** composed of mucoid polysaccharides or polypeptides. Capsule production can increase virulence in some microbes, such as *Streptococcus pneumoniae*, where the capsule makes the cells less vulnerable to phagocytosis by host immune cells. The capsule produced by *Streptococcus mutans* allows the cells to adhere to tooth enamel and contributes to plaque formation. The acids produced by bacteria trapped in the plaque can erode the enamel, which leads to the formation of dental caries. The nature of the capsule makes it difficult to stain.

In this exercise, you will learn and practice the proper technique for preparing a negative stain. Bacterial morphology will be observed microscopically under oil-immersion.

LABORATORY EXERCISE

5.1 Negative Stain.

Each student or laboratory group will prepare a negative stain using the organisms below as assigned by the teaching assistant.

ORGANISMS

☑ *Bacillus megaterium*
☑ *Paenibacillus velaei*

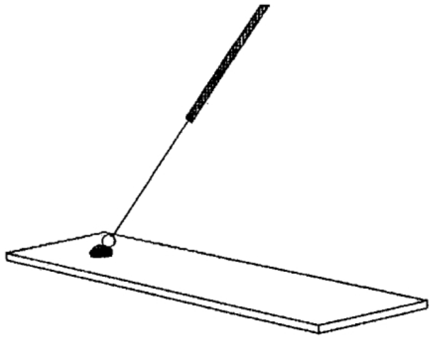
MATERIALS

☑ Nigrosin stain
☑ microscope slides
☑ inoculating loop
☑ immersion oil
☑ lens paper

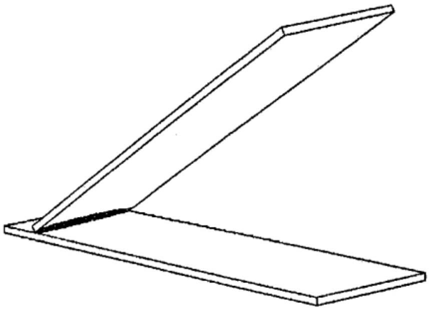
PROCEDURE

1. Place a small drop of nigrosin stain near one end of a clean microscope slide.
2. Using a sterile loop, obtain a sample of the assigned organism from the plate and place the organism into the drop of stain.
3. Hold a second clean slide at about a 45 degree angle to the first. Place the end of this slide into the drop of stain and slowly pull the stain across the surface of the slide toward the opposite end. This should result in a thin film of stain on the surface of the slide.
4. Allow the stain to air-dry completely.
5. Observe the thin areas of the stain microscopically under oil-immersion (see Exercise 1.3). Organisms appear clear against a dark background.

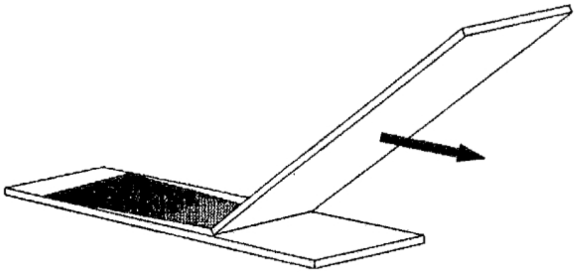
FIGURE 5.1 Negative staining technique.



1. Place a drop of nigrosin near one end of a clean microscope slide. Add one loopful of organism.



2. While holding at an angle, place end of second slide into the drop of nigrosin. The liquid will spread along the edge.



3. Pull top slide across the surface of the first. This will spread the nigrosin suspension. Air dry the thin film.

LABORATORY REPORT 5
NEGATIVE STAIN

Name: ______________________________________

RESULTS

5.1 Negative Stain.

Briefly describe and sketch the observations from each of negative stain below:

Bacillus megaterium

Paenibacillus velaei

QUESTIONS

1. In the negative stain, the bacteria appear _________________ against a ______ background.
2. The negative stain is a simple method for making observations of a bacterial cell's.

____.

3. The nigrosin stain does not enter the cell because both the stain and the cell membrane are ___________ ____ charged.
4. A bacterial capsule is composed of a mucoid layer of ____.

LABORATORY SIX

Smear Preparation

LEARNING OBJECTIVE

Students will be able to:

- successfully prepare a smear for bacterial staining

PURPOSE

Preparation of bacterial cells fixed to microscope slides. These bacterial smears can then be used in a number of staining methods.

INTRODUCTION

Many bacterial staining methods are useful for identification of structural features (such as flagella or endospores) or biochemical properties of the outer cell membrane or wall (Gram and acid-fast stains). These staining methods include several additions of reagents and subsequent washes. In order to successfully stain the cells, it is necessary for the bacteria to be fixed to the slide so that they are not washed away during the procedure. Preparation of **bacterial smears** utilizes heat to fix the cells to the slide.

Smear preparations can be made using bacterial cultures from either liquid or solid media. The first few steps differ depending on which type of media is being used, but the remainder of the procedure is identical. For liquid media, a few loopfuls of culture can be placed directly on the slide and then spread using the loop to make a thin film across the center of the slide. A moderately turbid culture generally gives a good distribution of bacteria across the smear. When using samples from solid media, it is first necessary to place a small amount of water, again one or two loopfuls, on the slide. A small amount of culture can then be collected using a sterile loop and dispersed in the water. It is very important to use a minimal amount of culture (generally the amount obtained by gently touching the colony surface with the loop will be sufficient) as too many cells will produce a thick smear. Thick smears will not stain or destain evenly, producing unreliable results, and do not allow observations of individual cells due to crowding.

Another important consideration in preparing a successful smear is to completely air-dry the slide before applying heat. Any liquid that remains on the slide will produce steam during heating which will destroy the bacterial cells. Likewise, care must be taken during the heat-fix step of the dried smear. Failure to heat-fix will result in the cells being washed away during subsequent staining procedures. If the slide is heated too much, drastic size changes can occur in the cells.

In these exercises, you will learn the techniques for preparing bacterial smears from both broth and plate cultures. The smears will be used in later staining experiments.

LABORATORY EXERCISE

MATERIALS

☑ microscope slides
☑ wax pencil
☑ inoculating loop
☑ broth culture
☑ plate culture
☑ sterile water
☑ Bunsen burner

ORGANISMS

☑ *Escherichia coli*
☑ *Bacillus megaterium*
☑ *Staphylococcus epidermidis*
☑ *Micrococcus luteus*
☑ Other bacteria as assigned

PROCEDURE

6.1 Smear Preparation from Broth.

1. Make a circle in the bottom of the slide with a wax pencil to note where your organisms will be placed on the slide
2. Aseptically remove a loopful of organism from the broth culture. Make sure you cool the inoculating loop before it is inserted in the broth culture. This will prevent the formation of aerosols. Place the drop in the center of a clean and marked microscope slide.
3. Using the loop, smear the drop around the center of the slide to form a thin film.
4. Air-dry the film completely at room temperature.
5. Heat-fix the bacteria to the slide. This is done by holding the slide at one end and at right angle to the table top. Pass it slowly through the flame, then back again once. (The slide should not be too hot to hold. If it is, speed up the rate at which the slide is passed through the flame.)
6. Cool. The smear is now ready for staining.

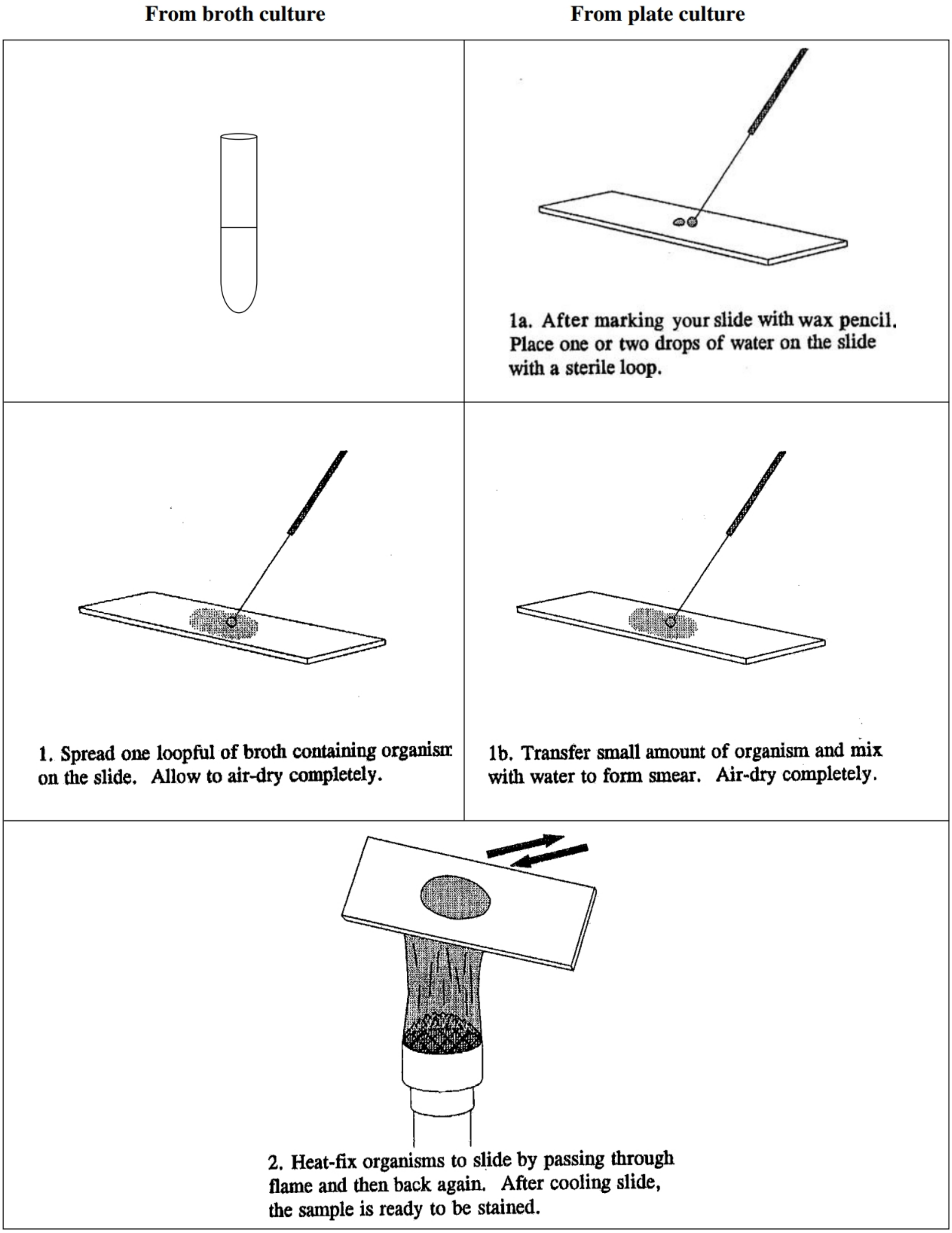
6.2 Smear Preparation from Plate Culture

1. Make a circle in the bottom of the slide with a wax pencil to note where your organisms will be placed on the slide
2. Place the 1–2 loopfuls of water in the center of a clean microscope slide.
3. Using a sterile loop, touch an isolated colony on the bacterial plate culture. Place the inoculated loop into the drop of water on the slide and mix.
4. Using the loop, smear the drop around the center of the slide to form a thin film.

Continue with Exercise 6.1, step 3.

Laboratory report at end of Laboratory 8

FIGURE 6.1 Smear preparation.



From broth culture

From plate culture

1a. After marking your slide with wax pencil. Place one or two drops of water on the slide with a sterile loop.

1. Spread one loopful of broth containing organism on the slide. Allow to air-dry completely.

1b. Transfer small amount of organism and mix with water to form smear. Air-dry completely.

2. Heat-fix organisms to slide by passing through flame and then back again. After cooling slide, the sample is ready to be stained.

LABORATORY SEVEN

Simple Stain

LEARNING OBJECTIVES

Students will be able to:

- successfully stain a smear with simple stain
- describe and distinguish different cell morphology types

PURPOSE

The simple stain is a rapid method for staining a bacterial smear.

INTRODUCTION

The simple stain is a rapid, easily-performed method for staining bacterial smears. A single basic stain, most commonly methylene blue or crystal violet, is used to colorize the cells in a few simple steps. Since the net charge of bacteria in aqueous solutions at neutral pH is negative, these basic dyes with their positively-charged chromophores (color-bearing ions) are attracted to the cell. In this manner, cells immersed in the stain take on the color. The simple staining method allows for general morphological observations of bacterial cells, but does not provide differential staining of cell types such as is possible with the Gram stain (see Laboratory 8).

In this exercise, you will stain a bacterial smear with a single, basic stain and observe the morphology of the cells microscopically under oil-immersion.

LABORATORY EXERCISE

7.1 Simple Stain.

ORGANISMS

(liquid culture in tube unless otherwise noted)

☑ Any bacterial culture.

MATERIALS

☑ Basic dye stain (ex. Methylene blue)

☑ microscope slides

☑ wax pencil

☑ inoculating loop

☑ immersion oil

☑ bibulous paper

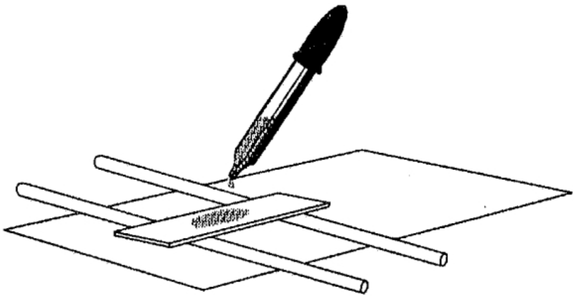
☑ lens paper

PROCEDURE

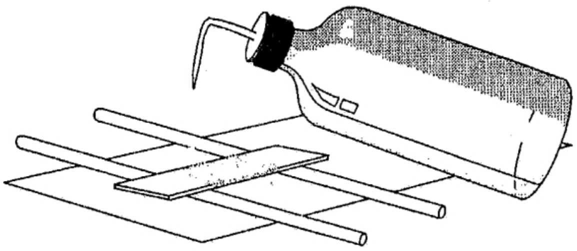
1. Prepare a bacterial smear (Exercise 6).
2. Cover the smear with the stain and leave for 1 minute.
3. Briefly rinse the slide with water. Blot dry with bibulous paper.
4. Observe under oil-immersion. The bacteria will appear colored against a clear background.

Laboratory Report at end of Laboratory 8

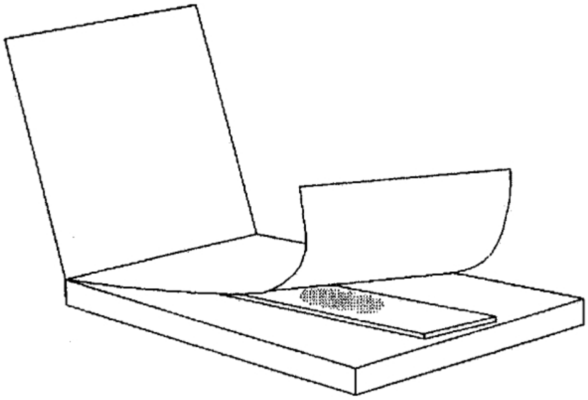
FIGURE 7.1 Simple staining technique.



1. Stain the heat-fixed smear with basic stain (*i.e.* methylene blue) for one minute.



2. Gently wash off the stain with water.



3. Blot the slide dry in bibulous paper. It may now be examined microscopically under oil-immersion.

LABORATORY EIGHT

Gram Stain

LEARNING OBJECTIVE

Students will be able to:

- successfully perform a Gram stain
- differentiate between a Gram positive and a Gram negative organism.

PURPOSE

The Gram stain is an important staining technique for differentiation of bacterial types. The purpose of this exercise is to learn the proper technique for preparing the Gram stain and to be able to correctly identify Gram-positive and Gram-negative organisms.

INTRODUCTION

The Gram stain was developed in 1884 by the Danish bacteriologist Christian Gram. This staining procedure is the most important differential technique applied to bacteria. The Gram stain divides bacteria into two groups, **Gram-positive** and **Gram-negative**, based on the ability of the cells to retain the purple color of **crystal violet** after decolorization with alcohol. The retention of the stain is based on structural differences between the cell walls of members of the two groups.

The Gram-positive cell wall consists of thick layers of peptidoglycan, a complex polysaccharide, outside the cell membrane. In the Gram stain reaction, an insoluble crystal violet-iodine complex is formed by application of a mordant, **Gram's iodine**, to the cells which have been stained with crystal violet, the primary stain. When the decolorizing agent **alcohol** is applied, the pores in the peptidoglycan layers of a Gram-positive cell become dehydrated and close. This traps the insoluble crystal violet-iodine complex in the cells and gives them a purple color.

The Gram-negative cell wall is more complex in structure, consisting of a relatively thin layer of peptidoglycan outside the cell membrane and then an additional outer membrane layer. The peptidoglycan and outer membrane together form the cell wall. When alcohol is applied to these cells, it easily penetrates the outer membrane and thin peptidoglycan layer. In this manner, the dye-iodine complex is removed from the cell. Since the cells would be colorless after this procedure, a counterstain, **safranin**, is applied to give the cells a red-pink color.

The Gram staining method is a valuable tool in the identification of bacterial unknowns. It is important when performing this procedure to always use fresh cultures, as old cultures of Gram-positive organisms will often decolorize more rapidly than young ones and will incorrectly appear Gram-negative. Along with the Gram-positive and Gram-negative bacteria, there are some groups of bacteria which stain poorly or not at all, such as *Mycobacterium*.

FIGURE 8.1 Change in cell color throughout the Gram staining procedure.

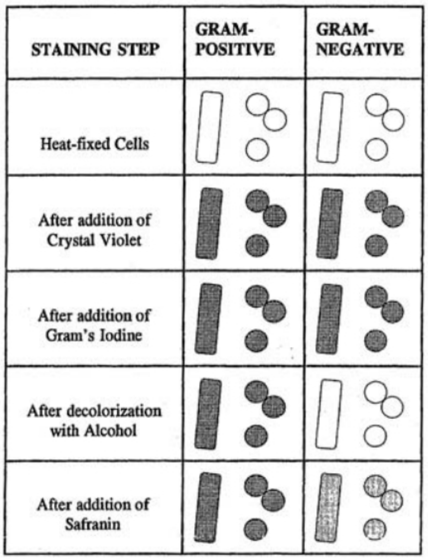
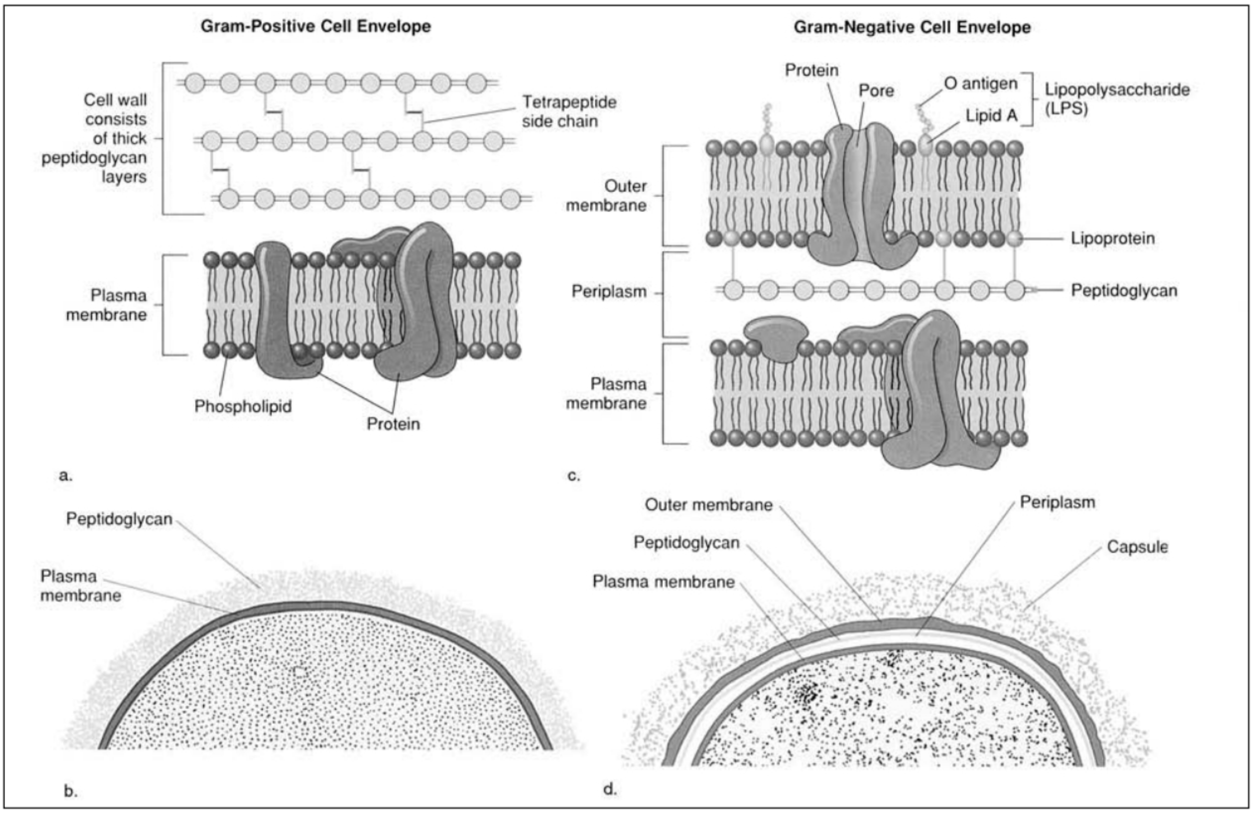

STAINING STEP
GRAM-POSITIVE
GRAM-NEGATIVE
Heat-fixed Cells
After addition of Crystal Violet
After addition of Gram's Iodine
After decolorization with Alcohol
After addition of Safranin

FIGURE 8.2 Structure of Gram-positive and Gram-negative cell walls.


Gram-Positive Cell Envelope
Cell wall consists of thick peptidoglycan layers
Tetrapeptide side chain
Plasma membrane
Phospholipid
Protein
a.
Gram-Negative Cell Envelope
Protein
Pore
O antigen
Lipid A
Lipopolysaccharide (LPS)
Outer membrane
Lipoprotein
Periplasm
Peptidoglycan
Plasma membrane
c.
Peptidoglycan
Plasma membrane
b.
Outer membrane
Periplasm
Peptidoglycan
Capsule
Plasma membrane
d.

Copyright © Kendall Hunt Publishing Company

In this exercise, each student will perform the Gram stain on both Gram-positive and Gram-negative bacteria in order to correctly identify each type. Due to the importance of this staining method in microbiology, it is imperative that you practice this technique until you can easily and consistently achieve the correct results. The teaching assistant will confirm your results and note his or her approval on the laboratory report.

LABORATORY EXERCISE

8.1 Gram Stain.

ORGANISMS

(liquid and plate culture of each)

☑ *Bacillus megaterium*

☑ *Escherichia coli*

☑ *Pseudomonas fluorescens/ P. putida*

☑ *Staphylococcus saprophyticus/ S. epidermidis*

☑ *Micrococcus luteus*

MATERIALS

☑ Crystal violet

☑ Grams iodine

☑ 95% Ethanol

☑ Safranin

☑ microscope slides

☑ inoculating loop

☑ immersion oil

☑ bibulous paper

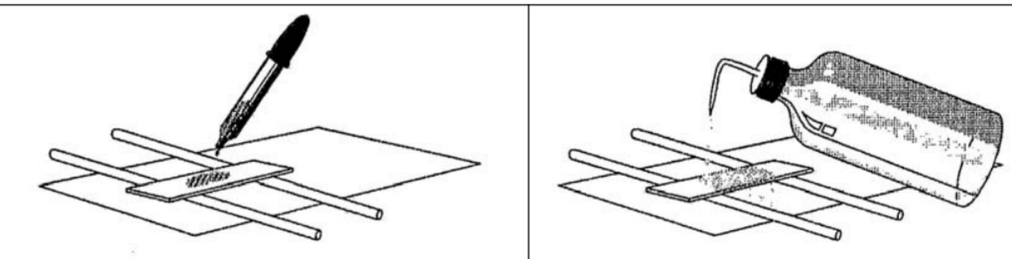
☑ lens paper

PROCEDURE

Each student will perform Gram stains on one Gram-positive organism and one Gram-negative organism from the list above. One of the bacterial smears should be made from a liquid culture and the other from a plate culture for practice in making each kind of smear.

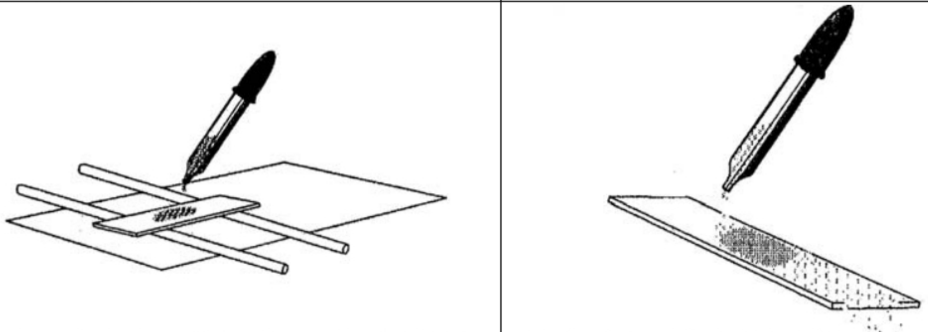
1. Prepare a bacterial smear (see Exercise 6.1).
2. Cover the smear with crystal violet and stain for 30 seconds to 1 minute.
3. Rinse briefly with water.
4. Cover the smear with Grams iodine. Stain for at least 1 minute.
5. Decolorize by rinsing with 95% ethanol for 5–10 seconds or until the color stops coming off the slide. Immediately rinse with water.
6. Cover the smear with safranin and stain for 30 seconds to 1 minute.
7. Rinse the slide with water. Blot dry with bibulous paper.
8. Examine under oil-immersion. Gram positive bacteria will be purple in color, while Gram negative bacteria will be red-pink. Have your teaching assistant confirm successful completion of the stains and note on the laboratory report.

FIGURE 8.3 Gram staining procedure.



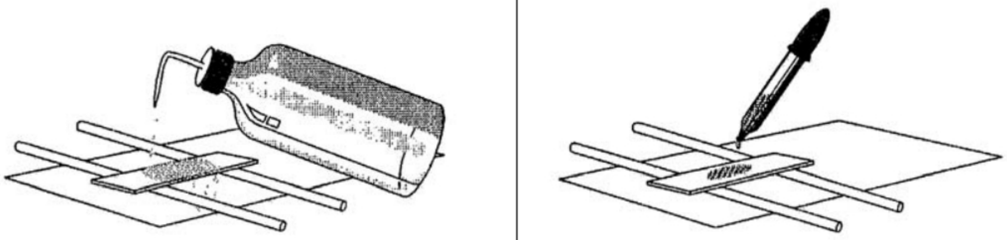
1. Add crystal violet to the heat-fixed smear. Stain for 30 seconds to 1 minute.

2. Gently rinse with water.



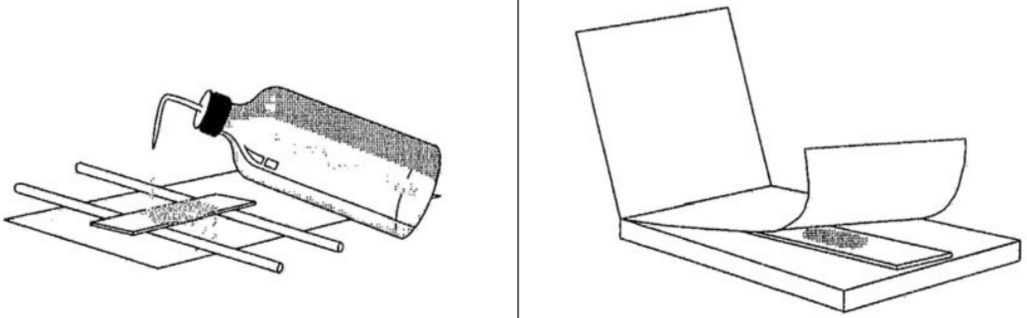
3. Cover the smear with Gram's iodine. Stain for at least 1 minute.

4. Decolorize with 95% alcohol for 5-10 seconds or until the color stops coming out of the smear.



5. Immediately rinse with water.

6. Cover the smear with safranin and stain for 30 seconds to 1 minute.



7. Briefly rinse with water.

8. Blot the slide dry in bibulous paper. It may now be examined microscopically under oil-immersion.

LABORATORY REPORT 6–8
BACTERIAL SMEAR, SIMPLE STAIN AND GRAM STAIN

Name:_______________________________________

RESULTS

7.1 Simple Stain.

Briefly describe and sketch the observations from the simple stained slide below:

8.1 Gram Stain.

Briefly describe and sketch the observations from the two Gram stained slides below (note colors):

Gram-positive organism: ___________________

This smear was made from a _____________ culture.

Teaching Assistant's confirmation: ________

Gram-negative organism: ___________________

This smear was made from a _____________ culture.

Teaching Assistant's confirmation: __________

QUESTIONS

1. It is necessary to __________ the smear so that the cells will be fixed to the slide.
2. It is only possible to make smears from broth cultures, not plate cultures. True or false.
3. The simple stain requires how many different stains? ______
4. The primary stain in the Gram stain is __________
5. The counterstain in the Gram stain is _________________
6. Alcohol is used to _________________ the sample.
7. The Gram stain distinguishes between structural differences in the _________________ of bacteria.
8. What color will a Gram-negative organism be at the completion of the Gram staining procedure? ______________
9. What is the purpose of the simple stain? _________________
10. What is the function of the mordant?_____
11. What is the difference in cellular structure of Gram-positive and Gram-negative bacteria? _________________
12. Which is the most critical step in the Gram-staining procedure? Why? _________________
13. Why is it important to use fresh cultures in the Gram-staining technique? _________________

LABORATORY NINE

Endospore Stain

LEARNING OBJECTIVES

Students will be able to:

- successfully perform an endospore stain
- Be able to identify endospores and distinguish between cells that do or do not contain endospores

PURPOSE

To differentially stain endospore-forming cells to distinguish the endospores within vegetative cells.

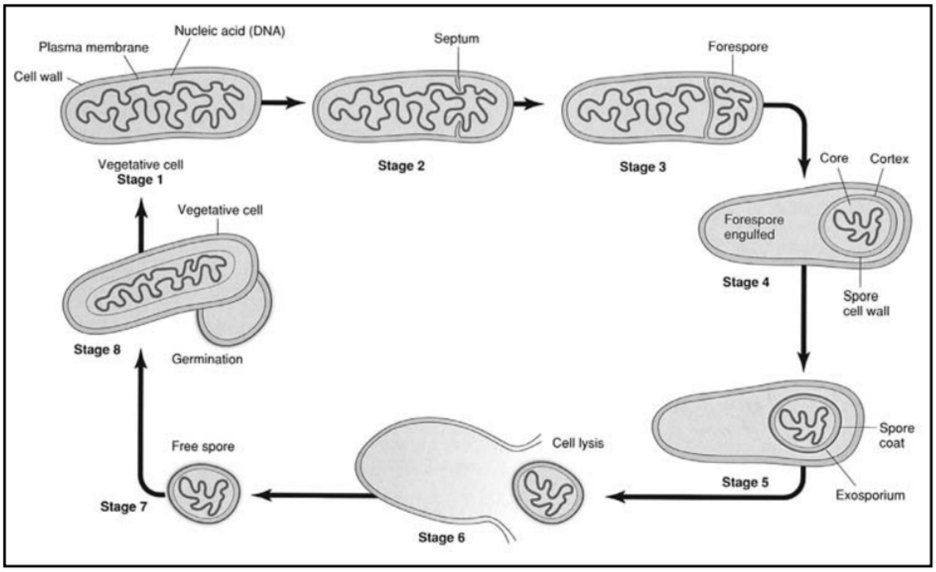
INTRODUCTION

The **endospore** is a specialized form of the bacterial cell which is dormant (Fig. 9.1). Endospores are formed by several bacterial genera, most notably *Bacillus* and *Clostridium*. This form of the cell is highly-resistant to a number of harsh environmental conditions, including heat, desiccation, and ultraviolet radiation. Endospores have a thick layer, known as the **spore coat,** which contributes to these resistant properties. While most are harmless soil organisms, several endospore-forming organisms are medically-important. These include *C. botulinum* which produces botulism toxin, *C. tetani* which causes tetanus, and *B. anthracis* which is the causitive agent of anthrax.

The spore coat also makes it difficult to stain the endospores. It is only possible to introduce stain into the endospores by applying the stain in the presence of heat. After staining a bacterial smear in this manner, the stain can be easily removed from the vegetative cells by rinsing with water, but remains in the endospores. The vegetative cells are then counterstained to give contrast.

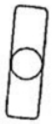
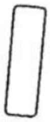
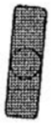
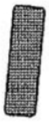
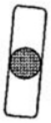
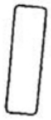
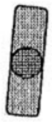
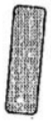
In this exercise, you will use the Schaeffer-Fulton method for staining endospores. This method uses **malachite green** as the primary stain and **safranin** as the counterstain.

FIGURE 9.1 Endospore cycle.


Nucleic acid (DNA)
Plasma membrane
Cell wall
Vegetative cell
Stage 1
Septum
Stage 2
Forespore
Stage 3
Core
Cortex
Forespore engulfed
Stage 4
Spore cell wall
Spore coat
Exosporium
Stage 5
Cell lysis
Stage 6
Free spore
Stage 7
Stage 8
Germination
Vegetative cell

Copyright © Kendall Hunt Publishing Company

FIGURE 9.2 Change in color during Schaeffer-Fulton spore stain.

STAINING STEP	SPORE FORMER	NO SPORES FORMED
Heat-fixed Cells		
After addition of Malachite Green and heat.		
After decolorization with water		
After addition of Safranin		

LABORATORY EXERCISE

9.1 Schaeffer-Fulton Method.

ORGANISMS

- ☑ 2–3 day plate with culture of *Bacillus megaterium*
- ☑ 7–10 day old plate culture of *Bacillus megaterium*

MATERIALS

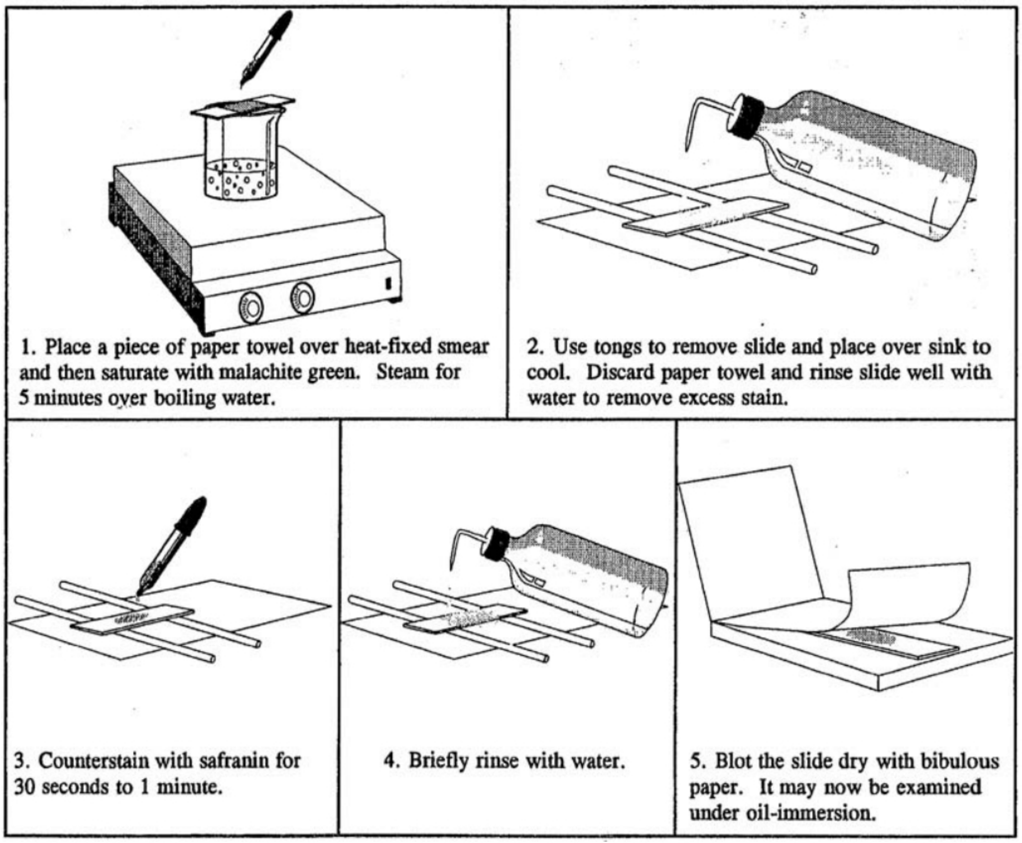
- ☑ Malachite green
- ☑ Safranin
- ☑ hotplate
- ☑ beaker
- ☑ microscope slides
- ☑ inoculating loop
- ☑ tongs or clothes pin
- ☑ immersion oil
- ☑ bibulous paper
- ☑ lens paper
- ☑ scissors
- ☑ paper towel

PROCEDURE

1. Prepare a bacterial smear (see Exercise 6.1).
2. Place the slide across a beaker containing boiling water. Position on the slide a small piece of paper towel cut to size which covers the area of the smear. Saturate the paper with malachite green and leave over the steam for 5 minutes. Add stain as necessary to prevent drying.
3. Remove the slide using tongs or clothes pin and place over sink to cool. Gently remove the paper towel piece and discard.
4. When cool, rinse the slide well with water.
5. Counterstain by covering the smear with safranin for 30 seconds to 1 minute.
6. Rinse the slide with water. Blot dry with bibulous paper.
7. Examine under oil-immersion. Endospores will be visible as green structures within red-pink vegetative cells.

Laboratory Report at end of Laboratory 10

FIGURE 9.3 Schaeffer-Fulton spore staining method.



1. Place a piece of paper towel over heat-fixed smear and then saturate with malachite green. Steam for 5 minutes over boiling water.

2. Use tongs to remove slide and place over sink to cool. Discard paper towel and rinse slide well with water to remove excess stain.

3. Counterstain with safranin for 30 seconds to 1 minute.

4. Briefly rinse with water.

5. Blot the slide dry with bibulous paper. It may now be examined under oil-immersion.

LABORATORY TEN

Acid-Fast Stain

LEARNING OBJECTIVES

Students will be able to:

- successfully prepare an acid fast stain
- identify acid fast organisms

PURPOSE

The acid-fast stain is a differential stain used to identify cells capable of retaining a primary stain after treatment with acid alcohol and is medically important in the identification of members of the genus *Mycobacterium*.

INTRODUCTION

The **acid-fast** stain is an important differential staining method in identification of members of the genus *Mycobacterium*. Some of the most important mycobacteria which can be detected in this manner are the *M. tuberculosis*, the causative agent in tuberculosis, and *M. leprae*, which causes leprosy. Most members of the genus contain large amounts of a waxy material, **mycolic acid**, in their cell wall.

The waxy layer makes mycobacteria difficult to stain. Since most aqueous stains are repelled by this layer, **carbolfuchsin** is used as the primary stain since it is a phenolic compound and thus lipid soluble. Once the cells have been stained with carbolfuchsin, they resist decolorization with **acid alcohol**. Because they retain the stain during this treatment, they are said to be acid-fast. Other bacteria in the sample are counterstained using **methylene blue**.

In this experiment, a non-pathogenic strain, *M. smegmatis*, will be stained using the Kinyoun acid-fast staining method. The acid-fast mycobacterium cells will appear as red bacilli, while bacteria which are not acid-fast will appear blue.

LABORATORY EXERCISE

10.1 Kinyoun Method – Cold Acid Fast Stain

ORGANISMS

☑ Plate culture of *Mycobacterium smegmatis*

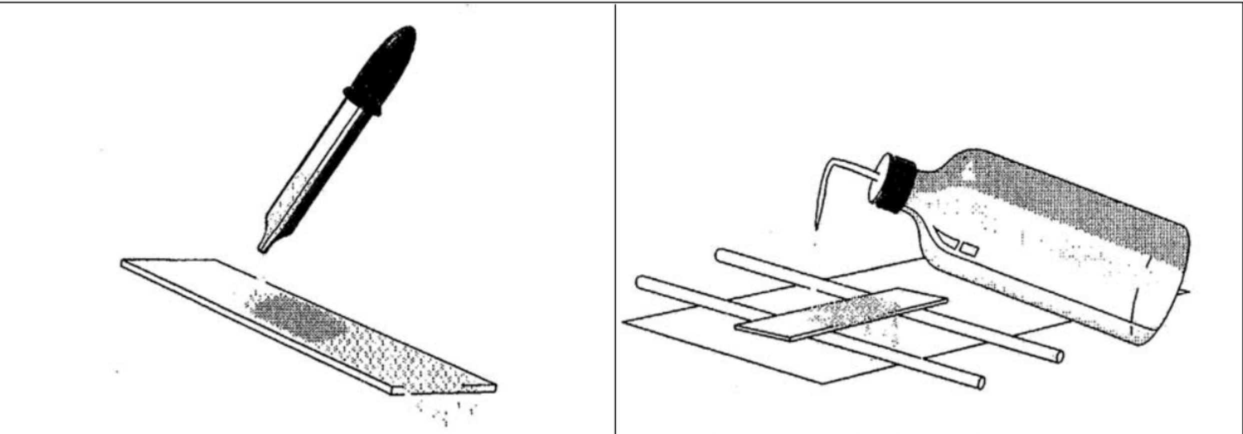
MATERIALS

☑ Carbolfuchsin
☑ Methylene blue
☑ microscope slides
☑ inoculating loop
☑ immersion oil
☑ bibulous paper
☑ lens paper
☑ small petri plates to collect saliva
☑ transfer pipette

PROCEDURE

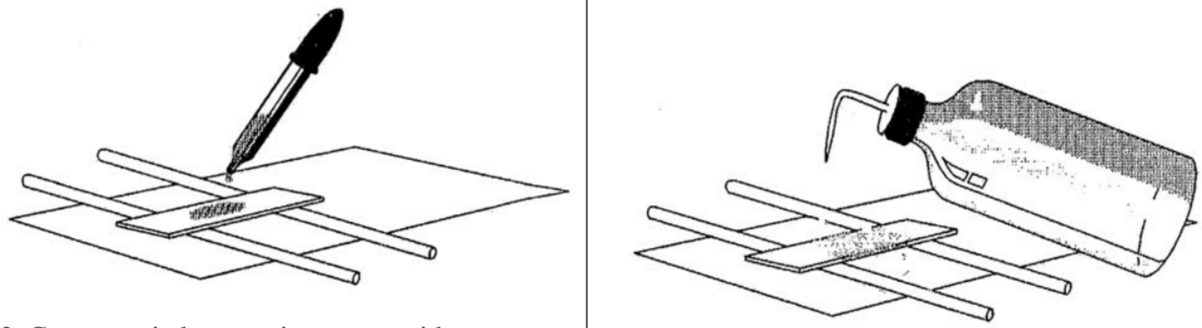
1. Prepare a bacterial smear (see Exercise 6.1), but modify preparation to use saliva instead of water to prepare the smear. Use loop to break up the clumps of *M. smegmatis*. The use of saliva will help to loosen the clumps, while also providing additional bacteria for comparison which are not acid-fast.
2. Place the slide over collection tray. Cover the slide with carbolfuchsin and leave for 5 minutes. Add stain as necessary to prevent drying.
3. Decolorize with acid-alcohol for 15-20 seconds.
4. Immediately rinse the slide with water.
5. Counterstain by covering the smear with methylene blue for 30 seconds to 1 minute.
6. Rinse the slide with water. Blot dry with bibulous paper.
7. Examine under oil-immersion. Acid-fast cells will be bright red in color, while non-acid-fast bacteria with be blue.

FIGURE 10.1 Kinyoun acid-fast staining method.



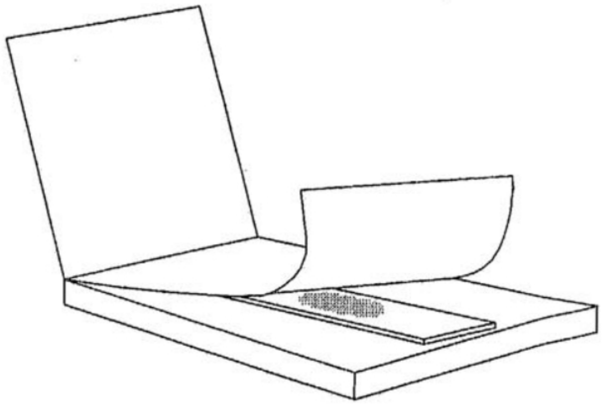
1. Add carbolfuchsin to the heat-fixed smear stain for 5 minutes. Decolorize with acid-alcohol for 15–20 seconds.

2. Immediately rinse with water.



3. Counterstain by covering smear with methylene blue for 30 seconds to 1 minute.

4. Briefly rinse with water.



5. Blot the slide dry with bibulous paper. It may now be examined microscopically under oil-immersion.

LABORATORY REPORT 9–10
ENDOSPORE STAIN AND ACID-FAST STAIN

Name: ___________________________________

RESULTS

9.1 Endospore Stain.

Briefly describe and sketch the observations from the endospore stained slide below (note colors):

2–3 day old culture

7–10 day old culture

10.1 Acid-fast Stain.

Briefly describe and sketch the observations from the acid-fast stained slide below (note colors):

QUESTIONS

1. The waxy outer layer found in acid-fast organisms is composed of __________________, which makes it more __________ to staining.
2. In the endospore stain, the primary stain __________________ penetrates the endospore.
3. __________________ is the decolorizing agent in the acid-fast stain.
4. Give the genus and species of a disease-causing, acid-fast organism: __________________
5. The __________________ method was used to stain the endospores.
6. The __________________ surrounds and protects the endospore.
7. The primary stain in the acid-fast stain is __________________.
8. The counterstain in the acid-fast stain is __________________.
9. What is the purpose of the counterstain in the acid fast stain? ________________
10. What is the significance of spore formation in food industry? ________________
11. How does the age of the *Bacillus* spp. cultures affect the spore staining results? ________________

SECTION THREE

Bacterial Growth and Control

LABORATORY ELEVEN

Bacterial Population Count

LEARNING OBJECTIVE

Students will be able to:

- Determine the amount of bacteria per milliliter in a bacterial growth culture

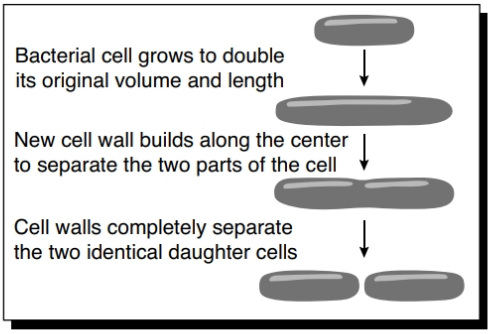
PURPOSE

- To learn techniques to measure bacterial counts

INTRODUCTION

While **growth** is the increase in the mass of protoplasm of an organism or population, **reproduction** refers to an increase in the number of individuals. It is important to note that cells may grow without reproduction or may reproduce without growth. Rates of growth and reproduction may be unequal in a population, resulting in variation of cell sizes.

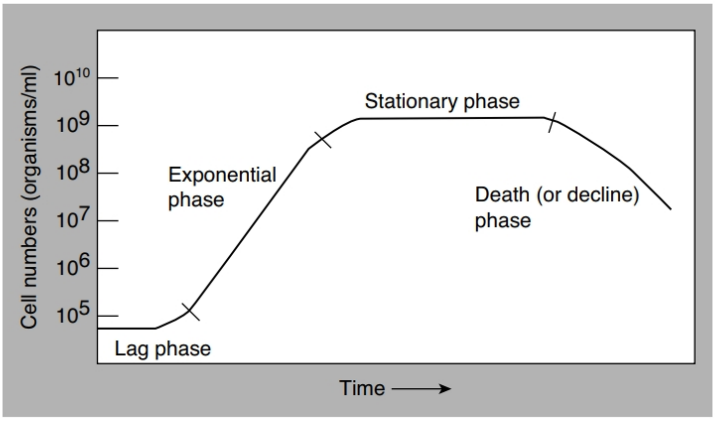
FIGURE 11.1 Bacterial division.


Bacterial cell grows to double its original volume and length
New cell wall builds along the center to separate the two parts of the cell
Cell walls completely separate the two identical daughter cells

In this laboratory, methods for bacterial population count will be performed. These methods are useful in determining the size of a bacterial population and can be used to monitor growth and/or reproduction within a population by sampling over time.

Measurement of growth

FIGURE 11.2 Growth curve.



1. Turbidity. As a beam of light passes through a suspension of bacterial cells, the light is scattered because of the difference in refractive index across the interface separating the cell from the medium. As a result, less light is transmitted by a tube containing a bacterial suspension than by the same tube filled with medium alone. The main factors which determine the amount of light scattered by the suspension are (1) the difference in refractive index between the cell and the medium, which depends on the concentration of solids, (2) size and shape of the cells, (3) the wavelength of light, and (4) the path length through the suspension.

One may measure the light scattered rather than the light transmitted. Measurement of scattered light is more sensitive than is measurement of turbidity. Instruments that measure scattered or reflected light are called **nephelometers.**

2. Determination of dry weight. An aliquot of the culture is centrifuged and washed free of the soluble constituents of the medium by further centrifugation from water. The washed cells are then dried to constant weight either at 105°C at ambient pressure or at lower temperature (e.g., 60°C) at reduced pressure.

3. Determination of some constituents of the cytoplasm. The washed cells can be analyzed for total N, organic P, or nucleic acid. If all constituents remain in constant proportions, the measurement of any constituent should suffice as a measure of growth.

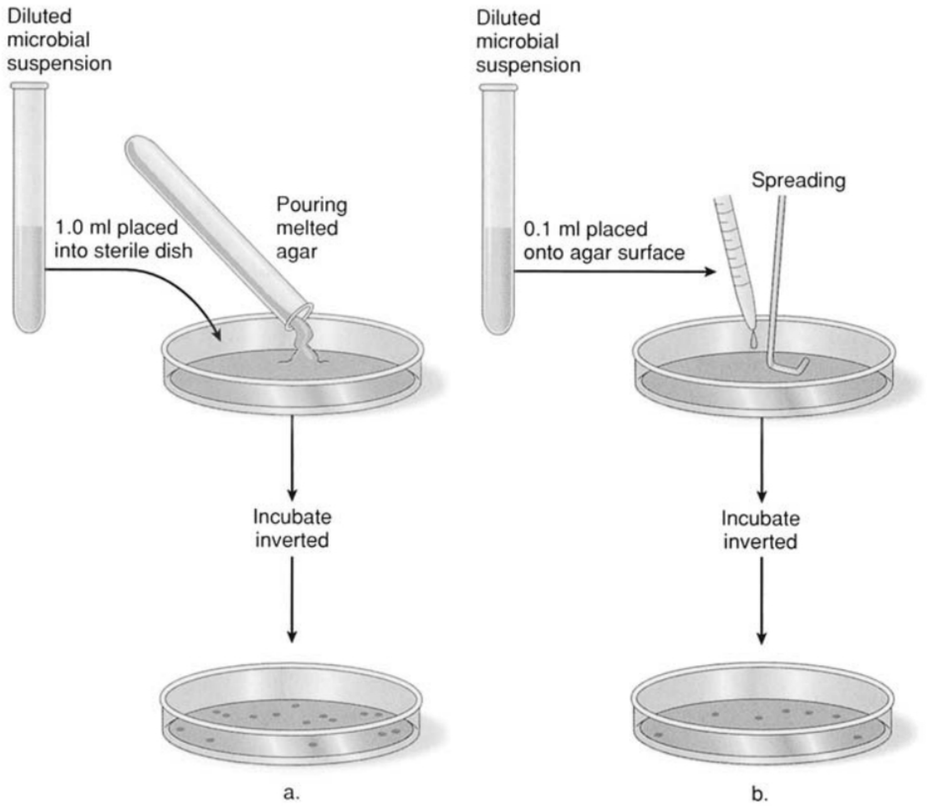
A variation of this technique is to supply an isotopic compound to the medium and determine the radioactivity in the cells after separation of the cells from the medium by filtration of a sample of the culture through a membrane filter.

4. Viable count is an estimate of the number of individual units which are capable of developing into a colony if placed in a suitable medium. A clump, a chain, or a single cell score equally. The method of viable counting depends on the physiology of the organism. Obligate or facultative aerobes are counted by spreading an aliquot of a suitable dilution on the surface of an agar medium.

5. Direct microscopic count. Direct counts can be made by placing a suitable dilution of the culture in a hemocytometer or Petroff-Hauser chamber. The cells are allowed to settle to the bottom of the chamber and are counted using a 4-mm (dry) phase contrast objective. Since the etched lines on the bottom of the chamber define known areas and since the depth of the chamber is known, the number of cells per unit volume can be computed.

Automatic particle counters obviously facilitate the direct counts of bacteria in suspension. Currently, the most popular is the Coulter counter which is based on a measurement of the

FIGURE 11.3 Pour and spread techniques for enumerating microorganisms.


Diluted microbial suspension
1.0 ml placed into sterile dish
Pouring melted agar
Incubate inverted
a.
Diluted microbial suspension
0.1 ml placed onto agar surface
Spreading
Incubate inverted
b.

Pour plate technique

Spread plate technique

Copyright © Kendall Hunt Publishing Company

electrical conductivity of liquid filling a small pore or aperture. The suspension is diluted such that the aperture is likely to contain at the most one bacterial cell, and a measured volume of the suspension is pumped through the aperture. If a cell enters the aperture, the conductivity is reduced. After the cell passes the aperture, the conductivity abruptly rises. The resulting electrical pulse is counted electronically.

TABLE 11.1 Summary of Methods for Measurement of Microbial Growth

Method	Use	Limitations
Direct microscopic count	Rapid laboratory enumeration of cell suspension	Requires large number ($>10^6$ cells/ml) of cells for accuracy
Viable count	Enumeration of viable cells in water, milk, and other products	Time consuming, requires proper medium for growth
Membrane filtration	Enumeration of bacteria from water, milk, and other products, especially when numbers are low	Time consuming, requires proper medium for growth
Most probable number (MPN)	Enumeration of bacteria from water, milk, and other products	Time consuming, requires proper medium for growth, provides indirect estimate of numbers
Turbidimetric measurement	Rapid estimation of cell density in a suspension	Does not differentiate between viable and nonviable cells, provides only estimate of cell density, requires $>10^6$ cells/ml
Dry weight determination	Determination of cell mass for industrial or laboratory applications	Time consuming, does not differentiate between viable and nonviable cells
Cell activity measurement	Research applications to follow cell metabolism	Involves extensive preparatory time

HOW TO USE THE MICROPIPETTERS

The micropipetters are a valuable tool in microbiology laboratories, they make the job of the microbiologist a lot easier since volumes can be moved a lot faster, and also there is a lot less materials to be discarded since only small plastic tips are used. Micropipetters might also be more accurate than regular glass pipettes.

Micropipetters are used to measure small volume, They are automatic, and a lot of times the volume measured can be adjusted (changed).

They most used sizes are:

"P-1000"—for volumes between 0.2ml and 10ml. in 2*u*l increments. The first number is in red and is always 0. This should be multiplied by 10 to obtain the actual volume dispensed.

For example if the window shows 067, the volume to be dispense will be 670*u*l.

"P-200"—for volumes between 0.02ml (20*u*l) and 0.2ml (200*u*l) in 1*u*l increments

"P-20"—for volumes less than 0.2ml (< 20*u*l) in 1*u*l increments.

To use the micropipetter

a. Dial the desired volume by turning the volume adjustment knob.

b. Aseptically insert the end of the micropipetter into the sterile disposable tips, press firmly with a light twist and remove the tip from the box.

  a. Never touch the tips with your fingers. Use the correct tips for the micropipetter.

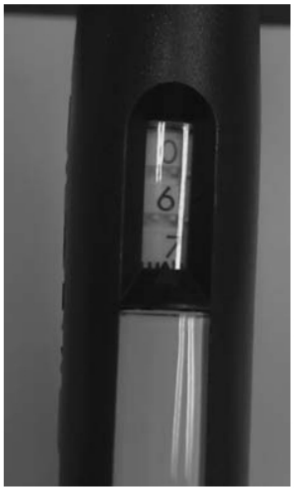
c. Using your thumb, slowly push down until you feel light resistance.

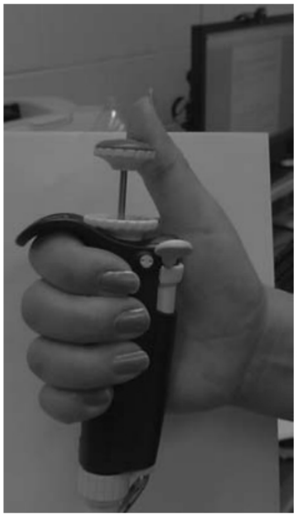
d. Immerse the disposable tip into the sample liquid to a depth of 1-2mm.

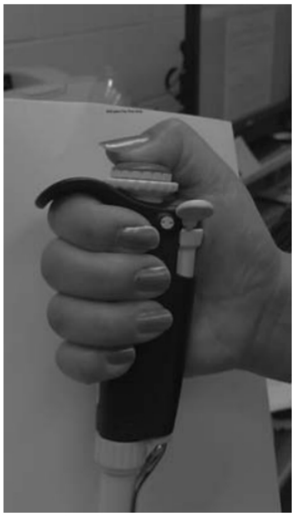
  a. Avoid jerky movements

e. Slowly allow the push button to return to its position.

f. Wait few seconds to make sure that all the volume was suck into the tip, withdraw the tip from your sample.







Images courtesy of Roxana Hughes

g. Dispense the liquid by gently pressing down on the button until your thumb will not go any farther. This will release the full volume of liquid from the pipette tip.

h. Discard the used tip in a biohazard container.

NOTES

- Take care of the micropipetters
- Do not force pass the volume of the micropipetter
- Place micropipetters in holder when not in use

EXAMPLES

“P-1000” [Top digit is red]		“P-200” all digits are black		“ P-20” the bottom digit is red	
1	0	2	0	2	0
0	6	0	5	0	4
0	7	0	2	0	5
1000*u*l	670*u*l	200*u*l	52*u*l	20*u*l	4.5*u*l

Practice

What volume will be pipetted? _________ml; ____________ul

“P-1000”		“P-200”		“ P-20”	
0	0	1	0	0	0
1	7	9	1	1	0
3	9	1	5	2	5

LABORATORY EXERCISE

11.2 Viable Cell Number.

ORGANISMS

(liquid culture in tube)

☑ *Escherichia coli*

SUPPLIES

☑ 2 dilution blanks, 9.9 ml

☑ 2 dilution blanks, 9.0 ml

☑ 4 tryptic soy agar (TSA) or nutrient agar (NA) plates

☑ micropipetter

☑ pipette tips

☑ turntable

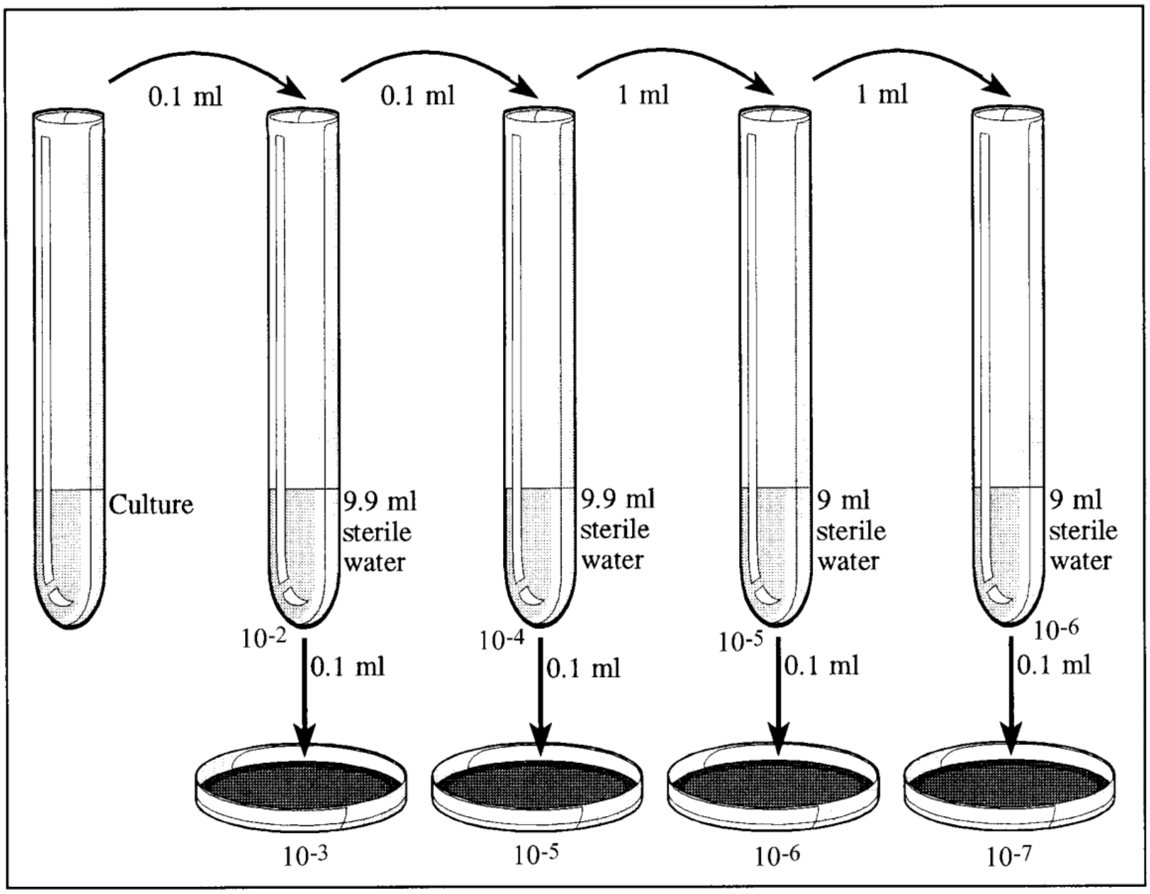
☑ cell spreader

☑ ethanol

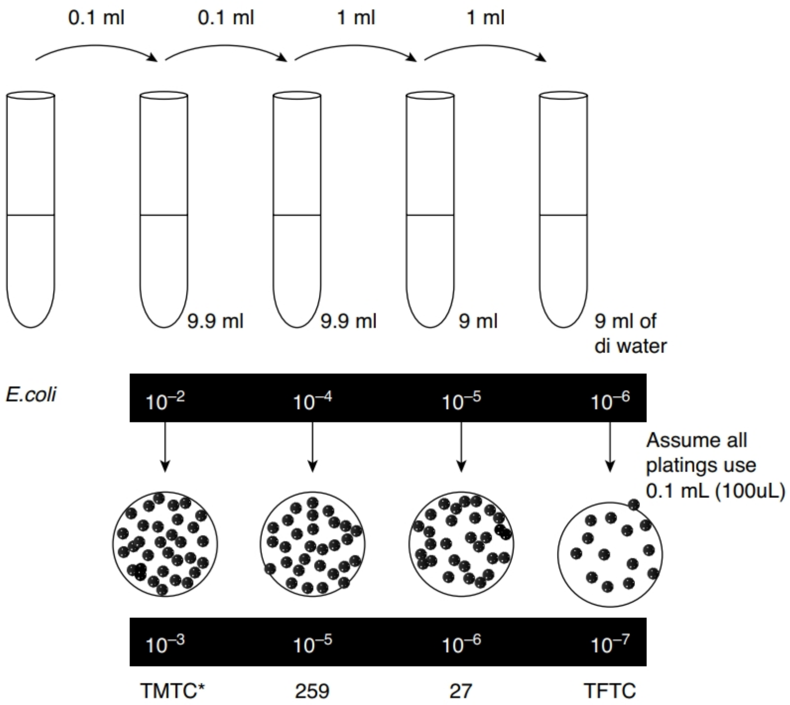
PROCEDURE

1. Obtain sterile dilution blanks (2–9.9 ml H_2O; 2–9.0 ml H_2O). Label them for serial dilution as follows: 10^{-2}, 10^{-4}, 10^{-5}, and 10^{-6}.
2. Transfer 0.1 ml of the *E. coli* culture to the tube labelled 10^{-2}. Mix well by vortexing. This is a 1:100 (10^{-2}) dilution.
3. With a fresh pipette, transfer 0.1 ml of sample from the first dilution tube (10^{-2}) to the second (10^{-4}). Mix well by vortexing. This is an additional 10^{-2} dilution and gives a total dilution at this point of 10^{-4}.
4. Repeat step 3 from the second dilution tube transferring 1.0 ml (10^{-4}) to the third (10^{-5}), and then transferring 1.0 ml from the third (10^{-5}) to the fourth (10^{-6}).
5. A 0.1 ml sample from each tube is plated to a TSA plate using the Spread Plate Technique (see Exercise 12.3). Since final bacterial counts will be per milliliter, it is necessary to account for the 0.1 ml sample which is plated. For the 10^{-2} dilution blank, the plate will be labelled 10^{-3}. The other plates should be labelled 10^{-5}, 10^{-6}, and 10^{-7} respectively.
6. Incubate the plates overnight at 37°C.
7. Observe the plates. Count colonies on plates which contain between 30 and 300 colonies. Calculate the Viable Cell Number (VCN) using the formula: VCN cell/ml = # colonies on plate X 1/dilution factor.

FIGURE 11.1 Serial dilution procedure.


0.1 ml
0.1 ml
1 ml
1 ml
Culture
9.9 ml sterile water
9.9 ml sterile water
9 ml sterile water
9 ml sterile water
10-2
10-4
10-5
10-6
0.1 ml
0.1 ml
0.1 ml
0.1 ml
10-3
10-5
10-6
10-7

Bacterial count practice (serial dilutions)


0.1 ml
0.1 ml
1 ml
1 ml
9.9 ml
9.9 ml
9 ml
9 ml of di water
E.coli
10^{-2}
10^{-4}
10^{-5}
10^{-6}
10^{-3}
10^{-5}
10^{-6}
10^{-7}
TMTC*
259
27
TFTC

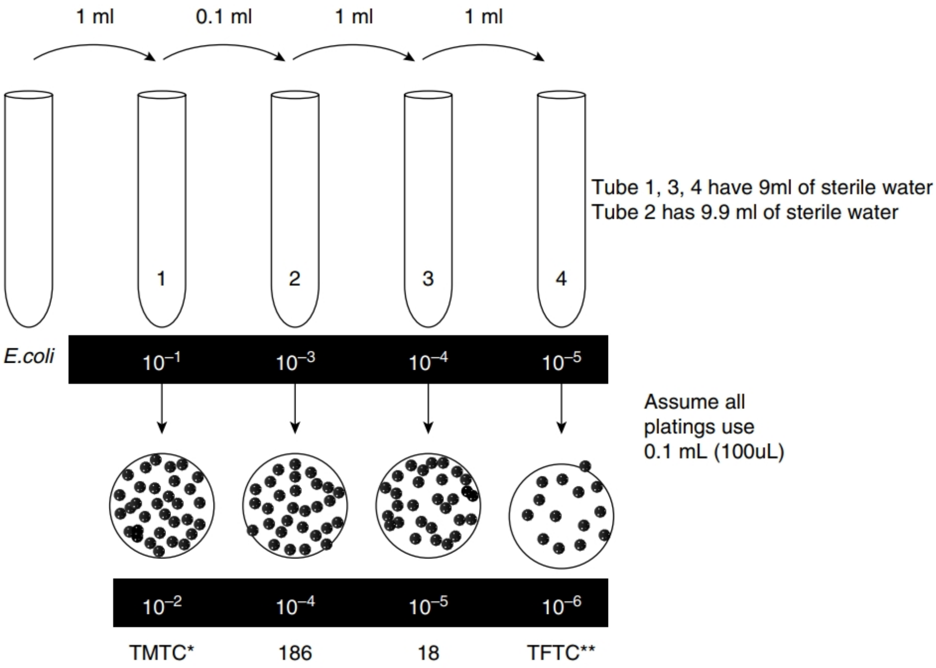
Assume all platings use 0.1 mL (100uL)

*TMTC = Too many to count
**TFTC = Too few to count

Choose a plate and calculate VCN for the sample (give proper units)

RESULTS

Viable cell number = VCN
VCN = # of colonies × 1/dilution factor
VCN = $259 \times 1/10^{-5}$
$= 2.59 \times 10^{7}$
so there are 2.59×10^{7} CFU/ml in the original sample


1 ml
0.1 ml
1 ml
1 ml
Tube 1, 3, 4 have 9ml of sterile water
Tube 2 has 9.9 ml of sterile water
1
2
3
4
E.coli
10^{-1}
10^{-3}
10^{-4}
10^{-5}
Assume all platings use 0.1 mL (100uL)
10^{-2}
10^{-4}
10^{-5}
10^{-6}
TMTC*
186
18
TFTC**

*TMTC = Too many to count
**TFTC = Too few to count

Choose a plate and calculate VCN for the sample (give proper units)

RESULTS

Viable cell number = VCN
VCN = # of colonies × 1/dilution factor
VCN = ____________CFU/ml

LABORATORY REPORT 11
BACTERIAL POPULATION COUNT

Name:______________________________________

RESULTS

11.1 Viable Cell Number.

Plate Dilution Factor # colonies

____

____

____

____

Calculate viable cell number (VCN) from the data:

$$\text{VCN} = \#\text{ of colonies} \times \frac{1}{\text{dilution factor}}$$

QUESTIONS

1. Compare results from the Viable Cell Number experiment with other lab groups. Are the results consistent? Explain your answer. ____

   ____

2. How do you determine which plate will give you the most accurate count?

   ____

3. Is Viable Cell Number an indirect or direct counting method?

   ____

4. The spectrophotometer measures the _______________________ of a sample to determine the amount of bacteria present.

5. The _________________________ chamber is used for direct microscopic counts.

6. Name one advantage and one disadvantage of the viable count method.

LABORATORY TWELVE

Temperature

LEARNING OBJECTIVES

Students will be able to:

- describe the effects of temperature on bacteria and bacterial enzymes
- describe the lethal effects of temperature on bacteria

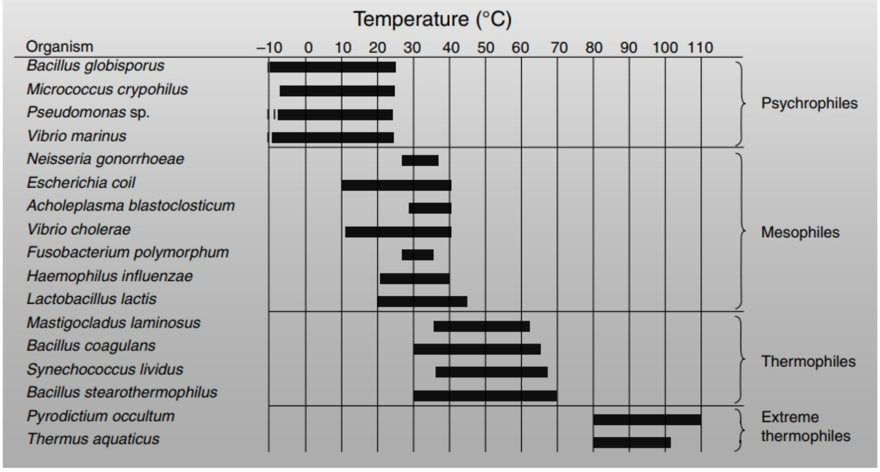
PURPOSE

The purpose of this exercise is to study the effects of temperature on enzymes, its effect on growth, and the effects of temperature on cell viability.

INTRODUCTION

Effect on Enzymes. In the life of a bacterium, temperature is one of the major influences on survival and quality of life. Life processes such as glycolysis, electron transport, DNA replication and protein synthesis, to name a few, rely directly on enzymes for the proper functioning of life-dependent biochemical reactions within the cell. Some transport of molecules in and out of the cell also takes place within the folded confines of enzymes which span the cell membrane.

An enzyme is almost always made up of protein (one exception is ribozyme, an enzyme made entirely of RNA) which is folded into a distinctive shape that binds to other substrates and facilitates chemical reactions. This unique folding pattern is important for the enzyme to find and bind to the right molecules. Temperature ranges for some psychrophiles, mesophiles, thermophiles and extreme thermophiles are shown below.



Copyright © Kendall Hunt Publishing Company

Therefore, it is critical that the enzymes remain at the optimal temperature for proper functioning. As temperature increases, chemical reaction rates increase. There is, however, a temperature threshold above which an enzyme no longer functions. At extreme temperatures, the protein which makes up enzymes becomes denatured and the enzyme unfolds making it dysfunctional or “killing” it altogether. Thus, there is a **maximal temperature** at which enzymes function. Likewise, there is a **minimal temperature** below which enzymes will not function. The **optimal temperature** is the temperature at which bacterial enzymes function best. The optimal temperature may differ among different types of organisms. There are three kinds of bacteria grouped according to their optimal temperature:

psychrophiles: low temperatures (5°C or below)

mesophiles: midrange temperatures (optimal range 30°C–37°C)

thermophiles: high temperatures (above 50°C)

At different temperatures, some enzymes which turn on pigment production may be activated. At 25°C, *Serratia* produces the red pigment, prodigiosin, but when grown at 37°C, there is no prodigiosin produced.

Effect on growth. The effect of temperature on bacterial growth is unique to each type of bacterium. There is a range within which bacteria will grow. What is a “low” temperature for one bacterium such as *Bacillus coagulans* (temperature tolerance of 25–65°C) is a “high” range temperature for *Bacillus globisporus* (temperature tolerance of –9°C to 25°C). Although some fungi and bacteria such as *Pseudomonas* can grow at lower temperatures in the refrigerator (around 4°C), the decrease in temperature retards growth of many microorganisms.

Control of microorganisms by heat. Complete sterilization using heat must be done in an autoclave at 121°C, at 15 pounds per square inch atmospheric pressure for at least 15 minutes. The combination of heat plus pressure kills bacterial endospores, the heat-resistant structures formed by a few bacteria (e.g. *Bacillus and Clostridium).*

Thermal Death Point:

- lowest temperature at which all bacteria in a broth culture are killed in 10 minutes.

Thermal Death Time:

- the minimum length of time for all the bacteria in a particular broth culture to be killed at a given temperature.

Decimal Reduction Rate:

- time in minutes in which 90% of a population of bacterial at a given temperature will be killed.

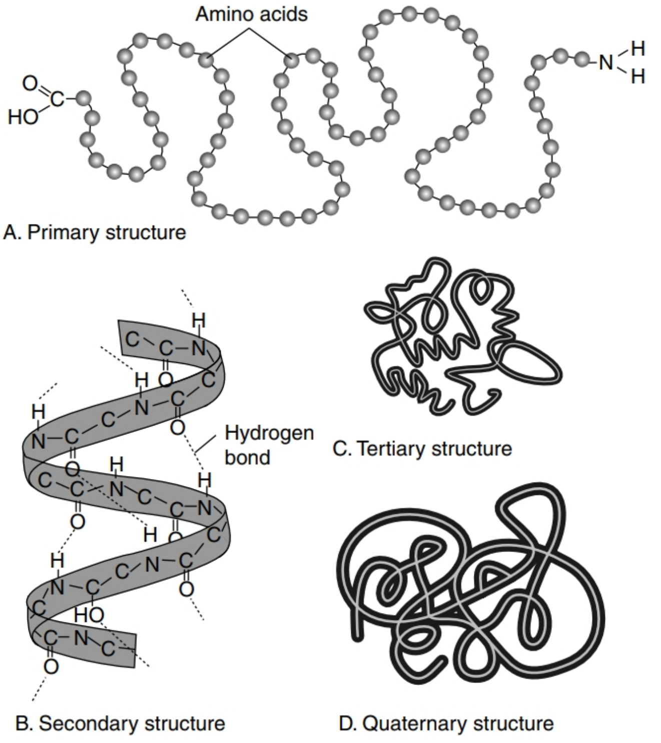
LABORATORY EXERCISE

ORGANISMS

(liquid culture in tube unless otherwise noted)

☑ *Bacillus megaterium*
☑ *Bacillus stearothermophilus*
☑ *Escherichia coli*
☑ *Serratia marcescens*

FIGURE 12.1 Protein structure.


Amino acids
A. Primary structure
Hydrogen bond
B. Secondary structure
C. Tertiary structure
D. Quaternary structure

Copyright © Kendall Hunt Publishing Company

12.1 Effect on Enzymes.

MATERIALS

☑ 2 nutrient agar slants
☑ incubators at 25°C and 37°C
☑ inoculating loop

PROCEDURE

1. Inoculate two slants with *Serratia marcescens*.
2. Incubate one slant at 25°C and the other at 37°C for 48 hours.
3. Observe growth and color.

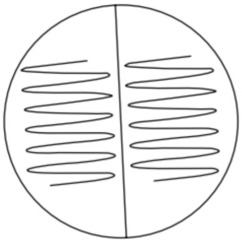
12.2 Effect on Growth.

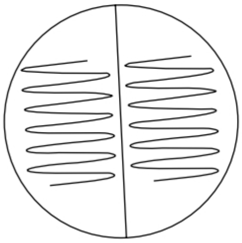
SUPPLIES

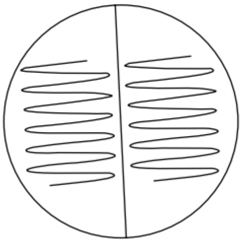
☑ 4 NA plates
☑ incubators at 4°C, 25°C, 37°C, and 55°C
☑ inoculating loop

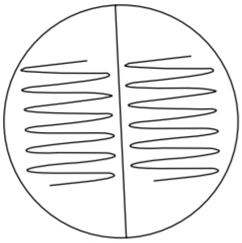
PROCEDURE

1. Inoculate four NA plates with *E. coli* and mark the plate into 2 halves, inoculate a streak of each culture with *Bacillus stearothermophilus*.
2. Incubate one NA plate with the organism at 4°C, at 25°C, at 37°C, and at 55°C for 24–48 hours.
3. Observe growth.


4°C


25°C


37°C


55°C

12.3 Lethal Effects.

MATERIALS

- ☑ 4 nutrient agar plates
- ☑ water baths at 60°C, 80°C
- ☑ beaker of boiling water (100°C)
- ☑ turntable
- ☑ hockey stick (cell spreader)
- ☑ alcohol
- ☑ 100 μl micropipetter
- ☑ test tube tongs
- ☑ Tips

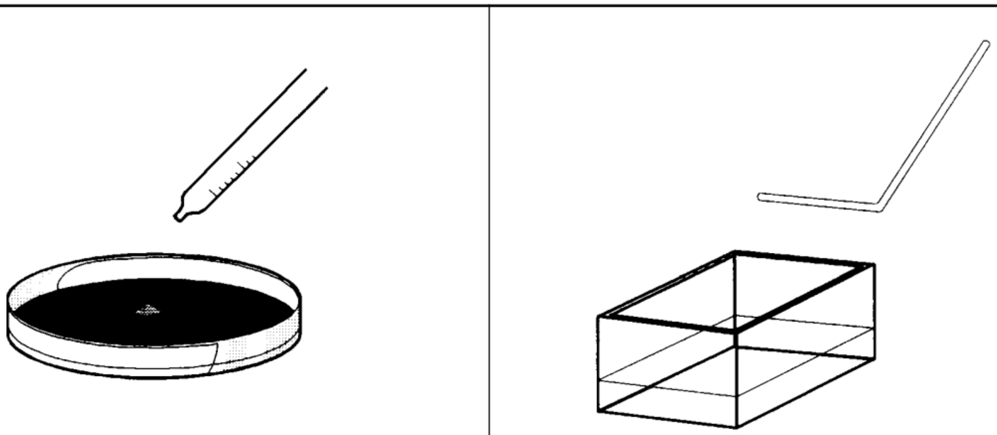
PROCEDURE

1. Each group will be assigned either *Escherichia coli* or *Bacillus megaterium* and one of the three temperatures by the teaching assistant. Using the assigned organism and temperature, prepare a spread plate (see below) on a nutrient agar plate for each of the following timepoints: 0, 10, 20, and 40 minutes. The zero timepoint sample must be done prior to placing the culture at the experimental temperature.
2. Place the culture tube at the assigned temperature and leave there when not taking samples.
3. After spreading, incubate plates at 37°C for 24 hours.
4. Observe growth and compare at different times and temperatures. Combine and discuss class results.

Spread plate technique.

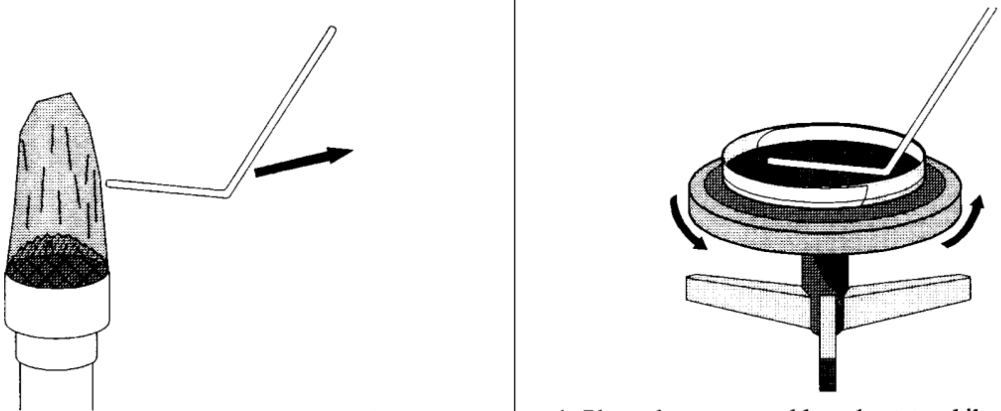
1. Pipette 0.1 ml of culture broth on agar surface.
2. Dip "hockey stick" in ethanol. Light ethanol in flame to surface sterilize (don't leave in flame because glass will overheat).
3. Place plate on turntable and rotate while spreading liquid with hockey stick. Hold lid over plate and work next to flame in order to minimize contamination.
4. Dip hockey stick in ethanol and flame again to sterilize. Allow plate to dry briefly before inverting for incubation.

FIGURE 12.1 Spread plate technique.



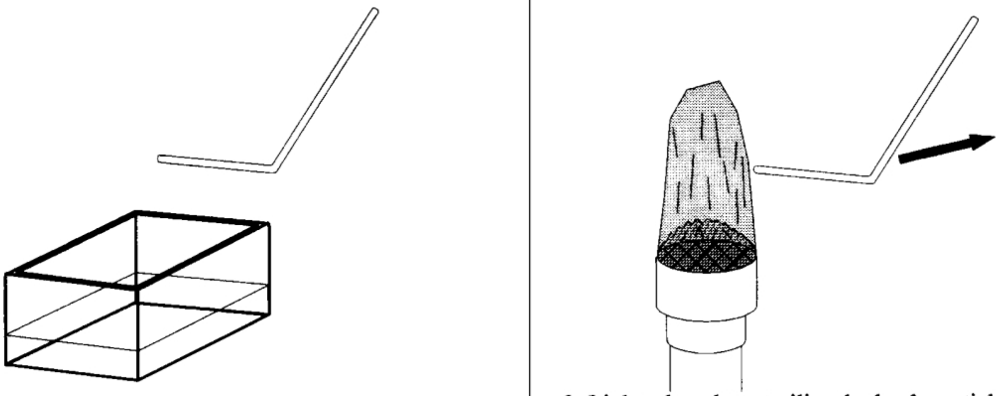
1. Pipette 0.1 ml of culture broth on to agar surface.

2. Dip hockey stick in ethanol.



3. Pass hockey stick through flame once to light the ethanol and sterilize the glass surface.

4. Place plate on turntable and rotate while spreading liquid with hockey stick. Work near flame.



5. Replace lid on plate. Dip hockey stick in ethanol once again.

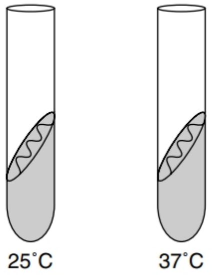
6. Light ethanol to sterilize the hockey stick. Let the plate dry briefly, then incubate inverted.

LABORATORY REPORT 12
TEMPERATURE

Name:_______________________________________

RESULTS

12.1 Effects on Enzymes



12.2 Effects of Temperature on Growth

	4ºC	25ºC	37ºC	55ºC
Escherichia coli				
Bacillus stearothermophilus				

12.3 Lethal Effects of Temperature

	0 minutes (control)			10 min			20 minutes			40 minutes		
	40/60	80	100	40/60	80	100	40/60	80	100	40/60	80	100
E.coli												
B.megaterium												

The organisms tested in my lab group was _______________________

The temperature used in my lab group was _______________________ºC

	Approximate Thermal Death Time	Approximate Thermal Death Point
Escherichia coli		
Bacillus megaterium		

QUESTIONS

1. Why is there a color difference in *Serratia marcescens* grown at the two different temperatures? ____

2. Was *E. coli* killed when incubated at 4°C? If yes, how? If no, what specifically prevented it from growing? ____

3. Which organism was more heat resistant in the Lethal Effects of Temperature experiment? Why? ____

Briefly define the following terms:

4. mesophile: ____
5. thermal death time: ____
6. thermal death point: ____
7. Organisms that grow at 0ºC and have optimum growth temperature of 15ºC or lower are called ____________________
8. Based on your class data- describe the follow-up experiment that would allow you to determine TDP (thermal death point) of *E.coli* ____
9. What are psychrotrophic organisms? What is their importance? ____
10. What is the optimum temperature of growth of most pathogens? Why? ____

LABORATORY THIRTEEN

Ultraviolet Radiation

LEARNING OBJECTIVE

Students will be able to:

- describe the effects of ultraviolet radiation on the growth of microorganisms

PURPOSE

To observe the lethal effects of ultraviolet radiation on microorganisms.

INTRODUCTION

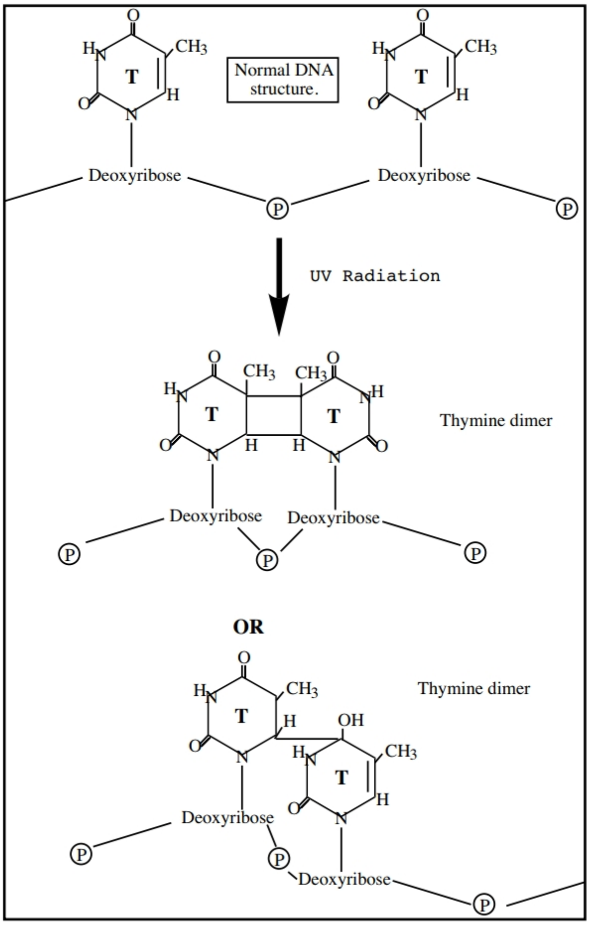
A **mutation** is a change in a base sequence of DNA which may be passed on to offspring. In bacteria, the mutation is expressed because the organisms are haploid and thus have only one gene expressing a trait. There are many agents that can cause mutations to occur, among them are chemical mutagens and ultraviolet (UV) radiation. In this exercise, the effects of UV radiation on the bacterial cell will be explored.

There are several forms of UV radiation that are mutagenic, but the one most commonly used in bacterial mutagenesis is nonionizing radiation (100–900 nm) which includes the UV range (100–400 nm). DNA damage occurs due to the absorbance of UV at 260 nm by pyrimidine and purine bases. The lethal effect of UV at 260 nm is due to the formation of pyrimidine dimers (Figure 13.1).

Since the geometry of the DNA double helix is distorted by the dimerization of pyrimidines, DNA replication cannot proceed faithfully. Since the DNA does not faithfully replicate itself, incorrect bases are inserted into the DNA helix and the organism dies or mutates.

In this experiment, we will examine the lethal effects and tolerance of different bacteria to UV exposure.

FIGURE 13.1 Pyrimidine (thymine) dimers produced in DNA after exposure to UV.



Normal DNA structure.
Deoxyribose
Deoxyribose
UV Radiation
Thymine dimer
Deoxyribose
Deoxyribose
OR
Thymine dimer
Deoxyribose
Deoxyribose

LABORATORY EXERCISE

13.1 Lethal Effects.

ORGANISMS

(liquid culture in tube unless otherwise noted)

☑ *Bacillus megaterium-24 hours*

☑ *Bacillus megaterium-1 week old*

☑ *Staphylococcus epidermidis*

☑ *Deinococcus radiodurans*

MATERIALS

☑ Nutrient agar plates/TSA plates
☑ UV lamp
☑ sterile cotton swabs
☑ index cards

PROCEDURE

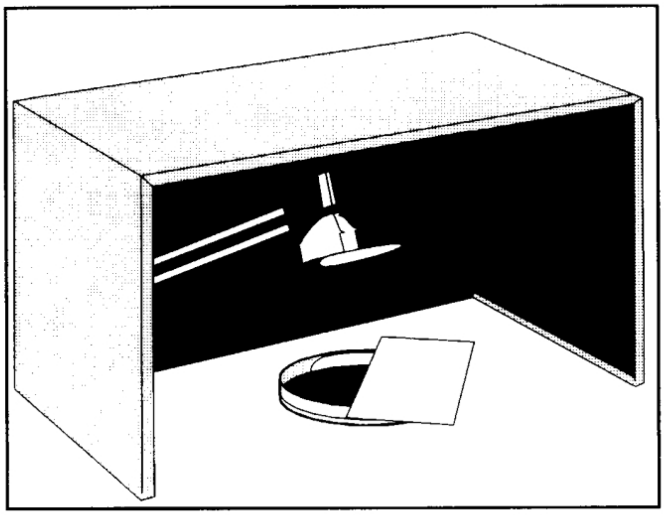
Each lab group will be assigned one of the organisms above and two or more of the exposure times for that organism.

1. Swab a rich plate with the assigned organism (one plate per timepoint) to make a bacterial lawn using the 3-way Swab Technique (see below).
2. Remove the lid and cover one half of the plate with an index card.
3. Immediately place the plate under the UV lamp at 260nm and leave for the assigned time (see below for time intervals of each organism). Caution: UV will damage unprotected eyes and skin. **Wear goggles and cover skin**. Place UV lamp under a box which is kept closed except when adding or removing plates.
4. Replace lid and incubate at 37°C.

Times of exposure

Organisms	**TIME 1**	**TIME 2**	**TIME 3**	**TIME 4**
Staphylococcus epidermidis	1 minute	5 minutes	10 minutes	15 minutes
Bacillus megaterium- 1 day old	1 minute	5 minutes	10 minutes	15 minutes
B.megaterium,- 1 week old	1 minute	5 minutes	10 minutes	15 minutes
Deinococcus radiodurans	1 minute	5 minutes	10 minutes	15 minutes

FIGURE 13.2 UV exposure plate.

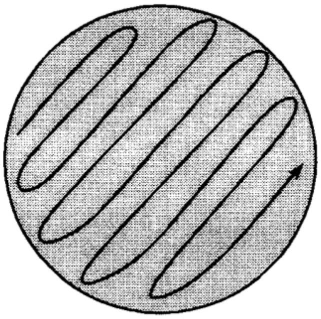


Note: Keep lids off the plates while exposing organisms to UV light.

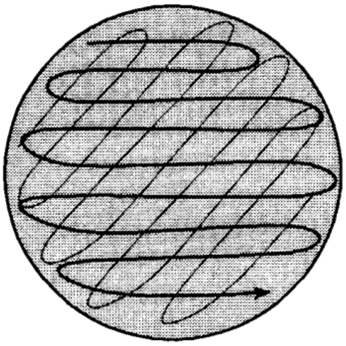
3-way Swab Technique

1. Inoculate a sterile cotton swab by aseptically dipping it into a broth culture.
2. Beginning on side of the plate, lightly brush the surface of the agar with the swab in a side to side motion. Swab the entire surface of the plate in this manner.
3. Rotate the plate a one-third turn and repeat the swabbing pattern with the same swab.
4. Complete the procedure by again rotating the plate a one-third turn and swabbing the surface as before. Incubate the plate at the appropriate temperature.

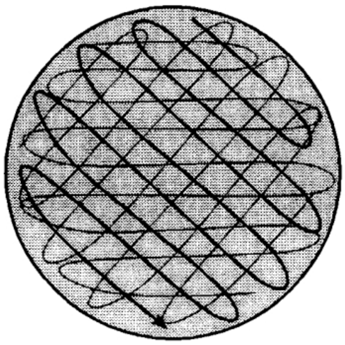
FIGURE 13.2 3-way swab technique.



1. Inoculate sterile cotton swab with broth containing organism. Swab the agar surface in one direction.



2. Rotate the plate by one-third. Swab the surface again using the same swab.



3. Complete the procedure by rotating the plate by another one-third and again swabbing the surface.

LABORATORY REPORT 13
ULTRAVIOLET RADIATION

Name:_______________________________________

RESULTS

Organism test by your lab group: _________________________________

Exposure times: ____

Sketch below the growth on the UV exposed plates:

Time 1 Time 2 Time 3 Time 4

Chart the class results:

Organism	**1 minute**	**5 minutes**	**10 minutes**	**15 minutes**
Staphylococcus epidermidis				
Bacillus megaterium- 24 hours-old				
Bacillus megaterium -1 week-old				
Deinococcus radiodurans				

QUESTIONS

1. The structure formed in DNA by UV exposure is the pyrimidine _____________.

2. Which are more resistant to UV, bacteria which form endospores or those which do not? ____________________

3. Of the genera tested in this experiment, which was the most resistant to UV exposure? ________________________

4. Name and briefly describe one method by which bacteria can repair DNA damage caused by UV irradiation:

   ____

   ____

   ____

5. At what wavelength is UV most harmful to bacteria? ____

   Why?____

   ____

6. Cite one disadvantage of the use of UV light as sterilizer. ____________________________________

   ____

7. Why did we have to cover half of the plate with an index card?

LABORATORY FOURTEEN

Oxygen Tolerance

LEARNING OBJECTIVES

Students will be able to:

- describe the different groups of bacteria based on tolerance to oxygen.
- determine which bacteria contain the catalase enzyme to break down toxic hydrogen peroxide to produce O_2.

PURPOSE

To study the role of oxygen in the growth of microorganisms

INTRODUCTION

Many organisms can tolerate toxic oxygen by production of enzymes that reduce its toxicity in the metabolism of superoxide radicals and hydrogen peroxide (see catalase test following).

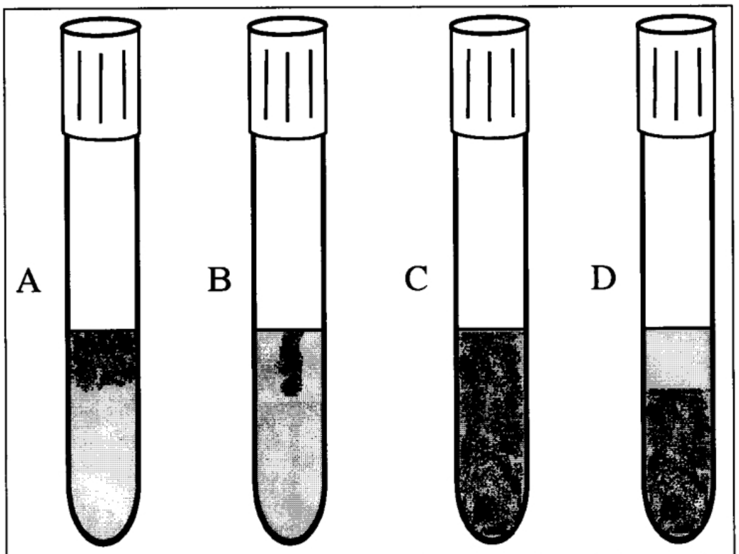
Organisms which *require* oxygen for growth are **strict (or obligate) aerobes** whereas those organisms which lack the enzymes to metabolize the toxic oxygen compounds are termed **strict (or obligate) anaerobes** (example, *Clostridium botulinum*). On the other hand, there are organisms which can grow either with or without oxygen and they are called **facultative anaerobes** (example, *E. coli*). Other types of oxygen-users are the **microaerophilic** bacteria which require low levels of oxygen in order to grow (*Enterococcus faecium*). These microaerophiles contain a peroxidase instead of catalase. **Aerotolerant** bacteria do not require oxygen, but growth is no better with or without oxygen.

To test bacteria for their tolerance of air, a growth medium which excludes air can be used. One such medium is **fluid thioglycollate** which contains a reducing agent that reduces oxygen to water. Oxygen can be absorbed only in the top portion of the tube containing the thioglycollate which comes in contact with air. The redox indicator dye, **resazurin**, is used as an indicator of air absorption in the medium by turning it a pink color. If thioglycollate tubes are totally or partially pink, they may be boiled to remove oxygen, and the tubes can once again be used to test for oxygen tolerance.

There are two methods to break down toxic hydrogen peroxide. Most aerobes and facultative anaerobes that tolerate and use oxygen in metabolic processes produce an enzyme called **catalase**. Catalase brings about the reduction of hydrogen peroxide to harmless water and oxygen gas as seen in Figure 14.3.

The substrate, hydrogen peroxide (H_2O_2), is added to colonies of organisms. As in the above reaction, if the organism produces catalase, it is able to reduce the hydrogen peroxide to water. One of the byproducts is oxygen gas thus producing the characteristic bubbling in a positive catalase reaction.

FIGURE 14.1 Growth patterns in fluid thioglycollate medium.

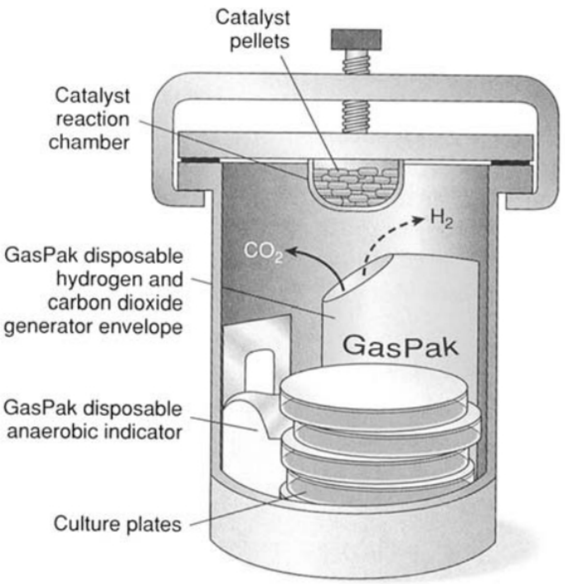


Pattern of growth in fluid thioglycolate medium for: A. aerobic organism; B. microaerophilic organism; C. facultative anaerobic organism; and D. anaerobic organism.

Culturing and Isolating Anaerobes

There are several methods to grow and culture anaerobes, anaerobic chambers, jars for anaerobic cultures, anaerobic boxes, and anaerobic bags (Becton Dickinson Bio-Bag)™. Any of these methods remove the oxygen present and allow the anaerobes to grow.

FIGURE 14.2 Anaerobic jar.


Catalyst pellets
Catalyst reaction chamber
H_2
CO_2
GasPak disposable hydrogen and carbon dioxide generator envelope
GasPak
GasPak disposable anaerobic indicator
Culture plates

Copyright © Kendall Hunt Publishing Company

FIGURE 14.3 Catalase test.

$$2O_2 + 2H^+ \xrightarrow{\text{superoxide dismutase}} H_2O_2 + O_2 + 2H^+ \xrightarrow{\text{catalase}} 2H_2O + O_2\uparrow$$

LABORATORY EXERCISE

ORGANISMS

☑ *Clostridium sporogenes* in fluid thioglycollate medium

☑ *Enterococcus faecium/ E. raffinosus*

☑ *Escherichia coli*

☑ *Pseudomonas fluorescens*

☑ *Staphylococcus* spp.

14.1 Oxygen Tolerance.

MATERIALS

☑ 4 Fluid thioglycollate medium tubes

☑ hotplate

☑ beaker

☑ test tube tongs

☑ inoculating needle

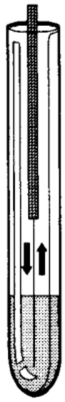
PROCEDURE

1. Inoculate one tube of fluid thioglycollate with each of *Enterococcus faecium, Escherichia coli, Pseudomonas fluorescens* and *Clostridium sporogenes* using an inoculating needle to make a stab.
2. Incubate all tubes at 37°C for 12–18 hours with the lids slightly loose.
3. Observe the growth pattern in the tube for each organism. Do not shake or otherwise disturb the tube. Note the presence of the zone of oxygen by the red pigment of resazurin.

Stabs.

1. Obtain a sample of culture on the sterile inoculating needle using the method described in Figure 14.4.
2. Open the tube to be inoculated and flame the mouth. Insert the inoculating needle with culture and stab straight down through the center of the agar plug. Pull the needle out of the agar in the same manner. Remove the needle from the tube, flame the mouth, and replace the cap.
3. Flame the needle to remove any residual culture. The inoculated stab may now be incubated at the appropriate temperature.

FIGURE 14.4 Stab technique**.**



1. Aseptically obtain culture on an inoculating needle. Stab the needle straight into the agar and remove in the same manner.

14.2 Culturing and Isolating Anaerobes

MATERIALS

- ☑ Tryptic Soy Agar plates
- ☑ Inoculating loop
- ☑ BIO-BAG (gas generators, catalyst, indicator)

ORGANISMS

- ☑ *Clostridium sporogenes*
- ☑ *Pseudomonas fluorescens or P.putida*

PROCEDURE

1. Preparing the Bio-Bag.
    a. Slide in the small brown cup (palladium) making sure it is lying flat
    b. Slide the resazurin indicator (arrow up, pointing toward opening of bag)
    c. Slide the large tube (weak HC and tablet of potassium borohydrate and sodium bicarbonate). Arrow up.
2. Inoculate the organisms (4th quadrant streak)
3. Sealing the Bio-Bag
    a. Quickly slide the inoculated plate into the Bio-Bag and take it to the heat-sealing device in the demo area (seal bags away from Bunsen burner flames)
    b. Flatten bag. Seal by pressing for ~4 seconds. Inspect seal
    c. Hold bag in the upright position
    d. Grab the middle of the small tube between your forefingers and thumb and squeeze until you feel/hear the glass ampule inside breaking (indicator turns to pink)
    e. Grab the gas generator and break (like above). When crushed, tap the gray tablet so that it falls down into the liquid released from the ampule. You should be able to see the liquid bubbling around the gray tablet (chemical reaction H_2-CO_2). Hold bag for 1 minute
    f. Label outside bag, incubate in basket at 37ºC

14.3 Catalase.

MATERIALS

☑ 3% hydrogen peroxide
☑ small petri dish
☑ Sterile tooth pick

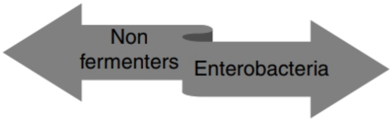
ORGANISMS

☑ *Enterococcus raffinosus*
☑ *Staphylococcus* spp
☑ *Bacillus megaterium*

PROCEDURE

1. Add a drop of hydrogen peroxide solution to the center of a petri dish.
2. Use the inoculation loop to aseptically transfer a visible portion of culture from a colony or slant to the hydrogen peroxide drop on the petri dish. Each students should test *Enterococcus* spp., *Staphylococcus* spp and *Bacillus megaterium*.
3. Observe. Catalase activity will be visible as bubbling in the solution caused by the release of oxygen gas.

14.4 Oxidase

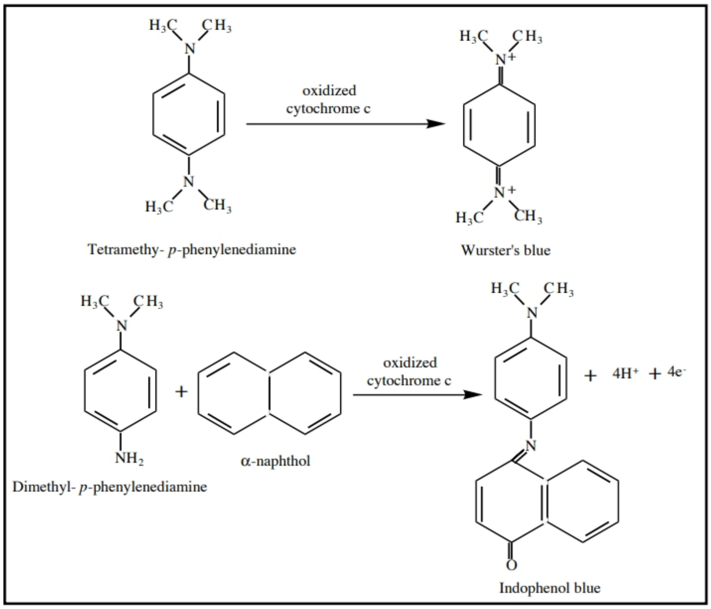

Non
fermenters
Enterobacteria

Cytochrome *c* oxidase is actually an enzyme complex made up of several different enzymes which pass ions down the electron transport chain within the bacterial cell membrane. Hydrogen ions and electrons are carried down the chain to the final electron acceptor (O_2) to produce water.

In the process of oxidation, ions are given up or "carried" to another "receiver enzyme" which becomes reduced (Lose Electrons Oxidation; Gain Electrons Reduction or LEO sez GER). Cytochrome *c* oxidase is an oxidizing agent; therefore, when bacteria containing cytochrome *c* are exposed to the oxidase indicator (tetramethyl-*p*-phenylenediamine or dimethyl-*p*-phenylenediamine), the reagent is oxidized and a black/purple color results.

Typically two different indicators may be used: tetramethyl-*p*-phenylenediamine or dimethyl-*p*-phenylenediamine. The reactions of each are in Figure 14.5. (Remember—this reaction takes place in the bacterial cell membrane.)

FIGURE 14.5 Oxidase Reaction.


oxidized
cytochrome c
Tetramethy- p-phenylenediamine
Wurster's blue
Dimethyl- p-phenylenediamine
α-naphthol
oxidized
cytochrome c
+ 4H+ + 4e-
Indophenol blue

ORGANISMS (plates)

- *Escherichia coli*
- *Pseudomonas fluorescens/P.putida*

PROCEDURE

1. Use a cotton swab to pick a well-isolated colony form a fresh bacterial culture
2. Add one drop of oxidase reagent (tetramethyl p-phenylenediamine) to the sample on the cotton swab.
3. Look for color changes to dark purple within 5 to 10 seconds. Disregard color changes after 60 seconds.

LABORATORY REPORT 14
OXYGEN

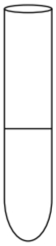
Name:____

RESULTS

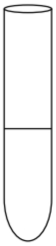
14.1 Oxygen Tolerance

Draw the tubes below for each organism tested. Indicate the zone of oxygen and the location of the growth for each.

C. sporogenes

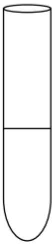


E. faecium/E.raffinosus



E. coli

P. fluorescens



14.2 Culturing and Isolating Anaerobes

Observe and describe the growth / no growth of the following organisms.

Clostrdium sporogenes: ____

Pseudomonas fluorescens or Pseudomonas putida: ____

14.3 Catalase.

Describe the results for each organism:

Enterococcus faecium/E. raffinosus: ____

Staphylococcus spp*:* ____

Bacillus megaterium: ____

14.4 Oxidase.

Describe the results for each organism:

Escherichia coli: ____

Pseudomonas fluorescens or P.putida: ____

QUESTIONS

1. An organism which can grow without oxygen, but which grows better in the presence of oxygen, is a(n) ________________________ ______________________.

2. The oxygen indicator in fluid thioglycollate medium is ______________________.

3. A ______________________________ requires oxygen for growth, but is killed by high concentrations of oxygen.

4. _________________________ is an enzyme which detoxifies hydrogen peroxide to water and oxygen gas.

5. An organism that grows on a plate that was incubated in anaerobiosis means that the organism is definitely anaerobe.

   a. True

   b. False

6. Name the indicator that anaerobic conditions were achieved in the BIO BAGS

7. What is the purpose of the palladium in the BIO BAGS

8. What is the purpose of the gas generator in the BIO-BAGS

9. What is the final concentration of oxygen in the BIO-BAGS

pH

LEARNING OBJECTIVE

Students will be able to:

- describe the effects of pH in the growth of microorganisms

PURPOSE

To observe the pH ranges at which bacteria and other microorganisms can grow.

INTRODUCTION

In microbiology, pH has to do with the alkalinity or acidity of a solution (either soil or aqueous) and its effect on the organisms growing therein. The classic definition of pH is the measurement of the hydrogen ion concentration. However, this means little until placed in the context of its effect on the life of an organism. All organisms have a certain pH range in which they flourish and grow. Indeed, the importance of the pH range for human beings is vital to the health of the individual both internally (pH of blood and body fluids) and externally (pH of foods eaten, cleaning solutions, etc). Some common pH ranges are shown in Table 15.1.

Microorganisms have been discovered growing at pH's ranging from 2 to 10 and even a few species have been isolated outside those ranges! At extreme pH's, life is limited to extremophiles, those bacteria which usually *require* an extreme pH in order to survive (*Thiobacillus* and archaebacteria, *Sulfolobus* and *Thermoplasma*.). Many fungi tolerate acid very well and grow at a pH of 5 or less but most bacteria which have been isolated grow better at pH ranges of 5 to 9.

In this experiment, extremes of pH tolerance will be investigated.

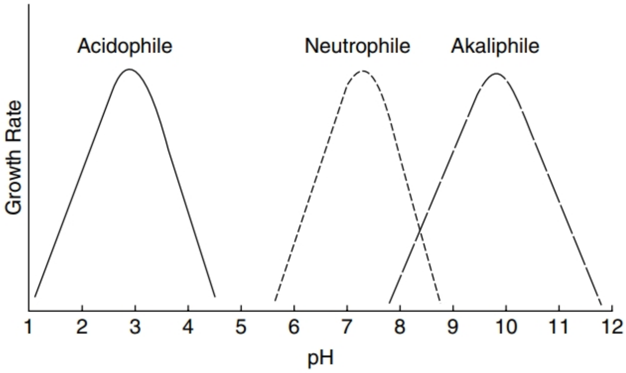


FIGURE 15.1 Common pH ranges. (Smoot & Price, 1975)

pH	EXAMPLE
1	HCl
1-2	Obligate acidophiles: *Thiobacillus, Sulfolobus*
2	Human gastric fluids
2.9	Vinegar
3	Rhubarb, peaches, soda pop
4	Acidic soil; tomatoes
4.5	Beer, growth of yeast
5	American cheese, cabbage, pumpkin, growth of fungi
6	Peas, corn, salmon, shrimp
6.6	Urine
6.9	Bile
7	Pure water, saliva, growth of many bacteria
7.4	Blood
7.5	Intestinal contents
8	Seawater
9	Alkaline soil and lakes
10	Soap solutions, soda lakes (growth of *Natronobacterium and Natronococcus*), high carbonate soils
11	Lime
13	0.1M NaOH

LABORATORY EXERCISE

15.1 pH.

ORGANISMS

(liquid culture in tube)

☑ *Saccharomyces cerevisiae*

☑ *Staphylococcus epidermidis*

☑ *Escherichia coli*

MATERIALS

- ☑ Nutrient broth, pH 5.0
- ☑ Nutrient broth, pH 7.0
- ☑ Nutrient broth, pH 10.0
- ☑ inoculating loop

PROCEDURE

1. Inoculate one broth tube at each pH for each of the organisms to be tested.
2. Incubate the bacteria at 37°C and the yeast at 30°C for 18–24 hours.
3. Observe and compare the growth characteristics for each organism. Note differences in amount and appearance of growth for an organism at the various pH's.

LABORATORY REPORT 15
pH

Name:______________________________________

RESULTS

Organism tested by your lab group: ______________________________

pH: Growth description:

____

Chart your group results:

Determine the optimum growth of the following organisms

	pH 5	pH7	pH10
Saccharomyces cerevisiae			
Staphylococcus epidermidis			
Escherichia coli			

QUESTIONS

1. An organism which prefers to grow at a low pH is called a(n) _________________________.
2. Define pH: ____.
3. What is the normal range of pH’s within which most bacteria grow? ___________________________.
4. As a solution becomes more acidic, the pH reading is ________________.
5. In the exercise, which organism would be expected to grow best at pH 5.0?

   ____

   Why? ____
6. Give an example of foods that are preserved when low pH are used.

LABORATORY SIXTEEN

Osmolarity

LEARNING OBJECTIVE

Students will be able to:

- describe the effects of different concentrations of sodium chloride on the growth of microorganisms.

PURPOSE

To observe the effect of water activity on the life of organisms.

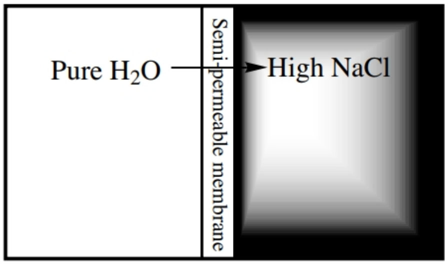
INTRODUCTION

Most life-processes take place in water, therefore the availability of water to an organism is of the utmost importance. Organisms which grow in a soil solution or in a liquid solution are affected dramatically by the concentration of solutes in solution. The solutes, such as sugars and salts, have a high affinity for water, making the water unavailable to the microorganisms.

Water diffuses from areas of lower solute concentration to areas of higher salt concentration in order to make the two concentrations of solute equal. In the illustration below (Fig. 16.1), water flows out of the pure water side on the left, through the semipermeable filter and into the area of higher salt concentration on the right, thus diluting the concentration of higher salt and equalizing the solute concentration. The process by which water diffuses across the membrane is called **osmosis**.

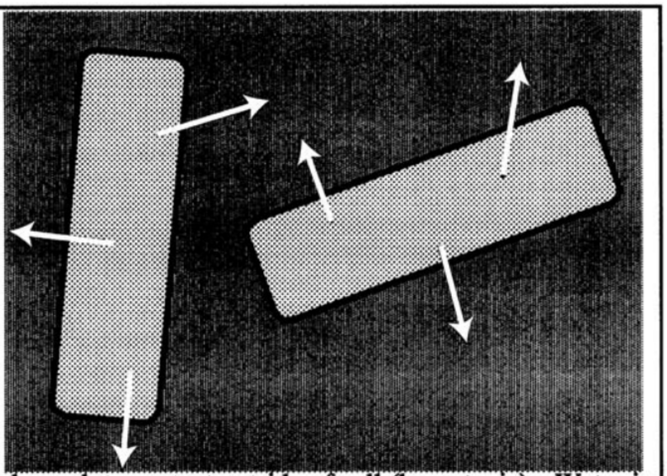
The cell membrane functions in much the same way as the semipermeable membrane above. The cell membrane allows water to diffuse across it when concentrations of solutes become unequal with that of the environment (Fig. 16.2). When an organism is placed in a solution

FIGURE 16.1 Diffusion of water across a semi-permeable membrane.

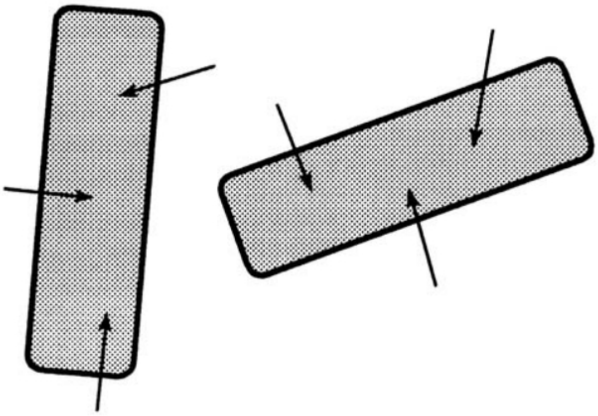


high in salt or sugar content (high solute concentration), it tries to equalize the solute concentration by losing water through its membrane. On the other hand, if the solute concentration is higher inside the cell than outside, water will diffuse *into* the cell to equalize the concentration.

FIGURE 16.2 Solute content relative to cell.



Higher solute content outside of cell (hypertonic). Water is lost through the membrane.



Lower solute content outside of cell (hypotonic). Water diffuses in through the membrane.

LABORATORY EXERCISE

16.1 Osmolarity.

ORGANISMS

(liquid culture in tube)

☑ *Escherichia coli*

☑ *Staphylococcus epidermidis*

☑ *Staphylococcus saprophyticus*

☑ *Halobacterium salinarum*

MATERIALS

☑ Nutrient agar plate with 0.5% NaCl

☑ Nutrient agar plate with 7.5% NaCl

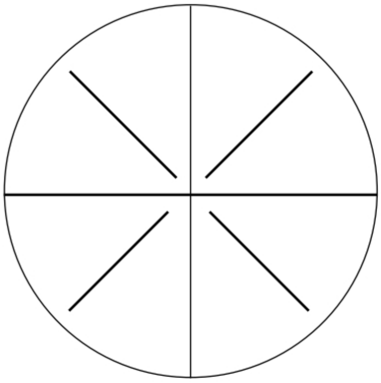
☑ Nutrient agar plate with 15% NaCl

☑ Nutrient agar plate with 25% NaCl

☑ inoculating loop

PROCEDURE

1. Obtain one each of nutrient agar plate with 0.5% NaCl, with 7.5% NaCl, and with 15% NaCl and 25% Nacl. Divide the plates into four quadrants by drawing lines on the bottom.
2. Label one selection of each organism on each plate. Make a single streak of each organism down the center of the appropriate quadrant.
3. Incubate the plates for 18–24 hours at 37°C.
4. Observe and record where growth occurred.



Obtain one NA plate of each NaCl concentrations. Divide in 4 quadrants. Inoculate each section with a single streak of each organisms.

LABORATORY REPORT 16
OSMOLARITY

Name:______________________________________

RESULTS

Record the growth of the organisms in different Nacl Concentration

	0.5% NaCl	7.5% NaCl	15% NaCl	25% NaCl
Staphylococcus saprophyticus				
Staphylococcus epidermidis				
Escherichia coli				
*Halobacterium salinarum**				

*Archaea

QUESTIONS

1. A ___________________________ solution has greater solute concentration relative to the cell.
2. An organism which *requires* high solute concentration is an _______________________ ___________________________.
3. Basesd on your results, does *Staphylococcus saprophyticus* require high solute concentration for growth? ____
4. _____________________ is the diffusion of water across a semi-permeable membrane.
5. In which direction will water move across the cell membrane of a bacterial cell placed into distilled water? ____
6. Define halotolerant ____
7. Define halophile

SECTION FOUR

Antimicrobials and Epidemiology

LABORATORY SEVENTEEN

Antiseptics and Disinfectants

LEARNING OBJECTIVES

Students will be able to:

- describe the effects of disinfectants/antiseptics on a lawn of bacteria
- describe the effects of antiseptic use on normal skin flora

PURPOSE

To demonstrate the effects of common antiseptics and disinfectants on bacterial growth through: 1. observing the effects of disinfectants/antiseptics on a lawn of bacteria; and 2. observing the effects of antiseptic use on normal skin flora.

INTRODUCTION

Both disinfectants and antiseptics are used to control bacterial growth, but in different situations. **Disinfectants** are usually much stronger in concentration and can be toxic to human beings as well as to microorganisms. **Antiseptics**, however, have been diluted sufficiently so as not to harm human beings. Some common antiseptics include organic mercurials, silver nitrate, iodine solution, 70% alcohol (EtOH in water) and hydrogen peroxide, among others. Examples of disinfectants include mercuric chloride, copper sulfate, iodine solution, chlorine gas, phenolic compounds, ethylene oxide and ozone.

There may be instances when the antiseptics and disinfectants are made ineffective when combined with organic materials which neutralizes the germicidal activity. Another reason antiseptics and disinfectants may not work effectively is that bacterial defenses may outperform the chemical agents. Some bacteria are encased in protective layers that prevent penetration of antimicrobial agents. Other bacteria produce spores which make the bacterium resistant to the antimicrobial agent.

Many antiseptics and disinfectants may not sterilize the skin or object being treated. **Sterilization** involves the complete removal/death of all living organisms. Many antimicrobial agents do not sterilize, but inhibit partial microbial growth.

In this laboratory exercise, the efficacy of common disinfectants/antiseptics will be tested by two methods, the plate evaluation and evaluation on skin.

LABORATORY EXERCISE

17.1 Plate Evaluation.

ORGANISMS

(liquid culture in tube)

☑ *Escherichia coli*

☑ *Pseudomonas fluorescens*

☑ *Staphylococcus epidermidis*

☑ *Bacillus magaterium*

☑ Another organisms as indicated

MATERIALS

☑ Nutrient agar plate

☑ Sterile disks of filter paper

☑ Forceps

☑ Alcohol

☑ Sterile swabs

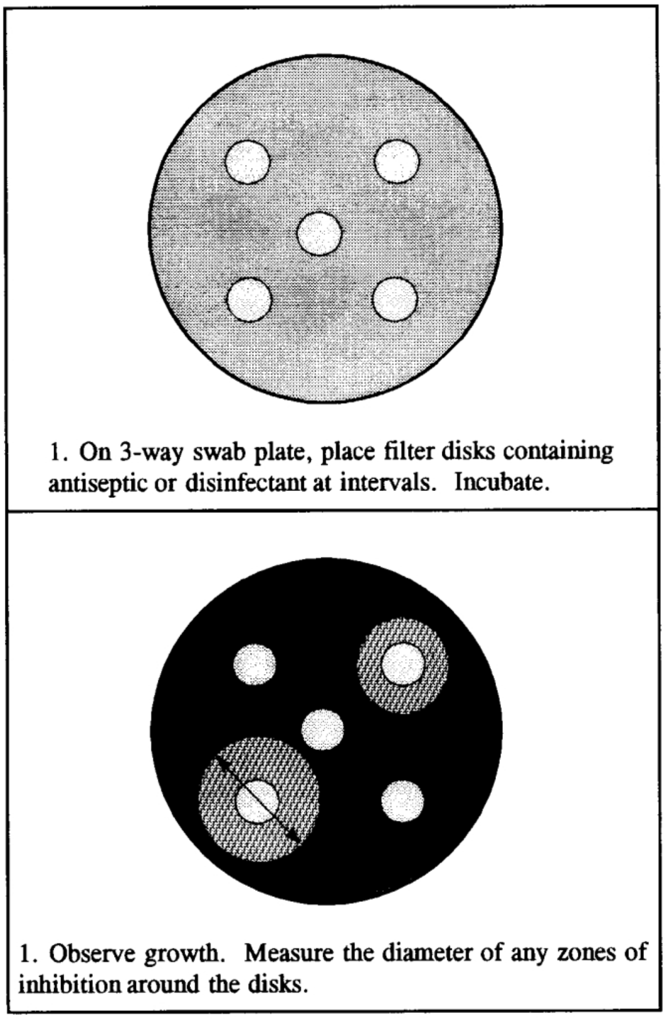
☑ Solutions of antiseptics or disinfectants (at working concentration)

☑ Ruler

PROCEDURE

1. Prepare a 3-way swab plate (see exercise 12.1) on nutrient agar for assigned organism(s). Allow to dry.
2. Using sterile forceps, dip a sterile filter disk in an antiseptic/disinfectant solution. Drain excess liquid.
3. Place the filter disk on the plate (allow space for 3-4 disks total). Repeat on same plate for all the solutions to be tested.
4. Incubate 18-24 hours at 37°C.
5. Observe the growth on the plate. Measure zones of clearing.

FIGURE 17.1 Antiseptic and disinfectant plate evaluation procedure.



1. On 3-way swab plate, place filter disks containing antiseptic or disinfectant at intervals. Incubate.

1. Observe growth. Measure the diameter of any zones of inhibition around the disks.

17.2 Evaluation on Skin.

SUPPLIES

☑ Nutrient agar plate

☑ alcohol or antiseptic swab

☑ antiseptic gel

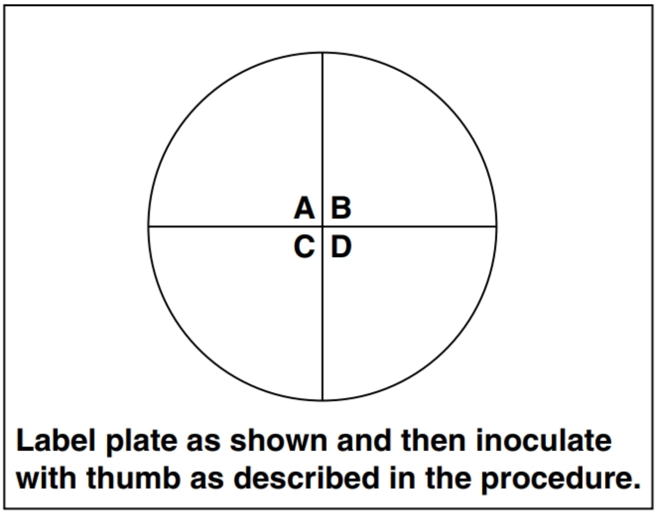
PROCEDURE

Note: Do not wash hands before beginning this experiment.

1. Obtain a nutrient agar plate. Divide the bottom into 4 quadrants labeled A, B, C, D.
2. Press unwashed left thumb gently on the surface of the agar in section A. Without touching any other surface, press the left thumb gently to section B. This is the experimental control.

3. Press unwashed right thumb gently on the surface of the agar in section C. The right thumb is then treated with alcohol swab (or antiseptic gel), allowed to dry, and then pressed gently to section D.
4. Incubate the plate for 18-24 hours at 37°C.
5. Count the number of colonies in each section (overlapping colonies each count as separate). The decrease in colony count from A to B serves as the control. Compare this to any decrease from section C to D. Differences greater than the control can be attributed to antiseptic action.

FIGURE 17.2 Setup for antiseptic evaluation on skin.


A
B
C
D
Label plate as shown and then inoculate with thumb as described in the procedure.

LABORATORY 17
ANTISEPTICS AND DISINFECTANTS

Name:_______________________________________

RESULTS

17.1 Plate Evaluation

Organism tested: __________________________________

Antiseptic/Disinfectant	Zone (mm)
_________________________	________
_________________________	________
_________________________	________
_________________________	________
_________________________	________
_________________________	________

Class results:

	Listerine	Lysol	Hydrogen peroxide	5% bleach	Iodine tincture	Other
S. epidermidis						
E. coli						
B. megaterium						
P. fluorescens						

17.2 Evaluation on Skin

# colonies on: A = ________, B = ________, C = ________, D = ________

A - B = _______

C - D = ________

QUESTIONS

1. A _________________________ is a compound used to sterilize inanimate surfaces.

2. In the plate evaluation, where in relation to the disk will the concentration of the compound be highest? ____________________

3. Why would a compound, such as soap, be ineffective in inhibiting bacterial growth on the plate evaluation, but be effective in decreasing bacterial numbers in the skin evaluation?

   ____

   ____

4. Were all the compounds tested in the plate evaluation equally effective against the range of organisms used? __________ What implications does this have for selection of antiseptics or disinfectants?

   ____

5. What is the action of the alcohol on the human skin?

LABORATORY EIGHTEEN

Antibiotic Susceptibility

LEARNING OBJECTIVES

Students will be able to:

- evaluate the effects of antibiotics on the growth of microoganisms
- determine the minimal inhibitory concentration of an antibiotic using the E-test method.

PURPOSE

To observe antimicrobial action of selected antibiotics.

INTRODUCTION

Anitbiotics and antimicrobial agents are compounds used to kill or inhibit growth of microorganisms. These are several different classes of antimicrobials with different mode of action on the bacteria. The major antibiotic actions are: inhibiting cell wall synthesis, inhibiting nucleic acids synthesis, inhibiting protein synthesis, causing plasma membrane injury, and affecting synthesis of essential metabolites.

An antibiotic can be bactericidal (killing of bacteria) or bacteriostatic (inhibition of bacteria).

One ofthe methods to test the susceptibility of bacteria to antibiotics is the Kirby-Bauer method. In this method, a Mueller-Hinton plate is spread with a lawn of a pure culture of bacteria, and antibiotic disks of predetermined concentrations are dropped onto the plate. After incubation, zones of inhibition are measured and compared against known ranges of inhibition for each drug.

Mueller-Hinton medium is the standardized medium for the antimicrobial disk susceptibility testing. It has controlled levels of tetracycline and sulfonamide inhibitors, magnesium, and calcium ions, are controlled and will not interfere with susceptibility testing.

The Minimal Inhibitory Concentration (MIC) of an antimicrobial agent is the measure of the smallest amount needed to inhibit growth of an organism. There are several different methods by which the MIC is determined. In this exercise the **E-test ™** ·(bioMerieux), in which plastic strips are impregnated with antibiotics in a **concentration gradient**, will be used.

Table 18.1 Interpretive standards for antimicrobial testing.

Disk Code	Antibiotic/ antimicrobial	Resistant (mm or less)	Intermediate (mm range)	Susceptible (mm or more)	Mode of action
CIP 5	Ciprofloxacin 5ug	15	16–20	21	Nucleic acids
E-15	Erythromycin 15ug	13	14–22	23	Cell wall
P 10	Penicillin 10 units				Cell wall
	Staphylococci	28		29	
	Enterococci	14		15	
S 10	Streptomycin 10ug	11	12–14	15	Protein synthesis
SxT25	Trimethoprim 1.25ug Sulfamethoxazol 23.75ug	10	11–15	16	Folic acid
TE 30	Tetracylcine 30ug	14	15–18	19	Protein synthesis
PB 300	Polymyxin B	8	9–10	11	Cell membrane

LABORATORY EXERCISE

18.1 Kirby-Bauer Method.

ORGANISMS

(liquid culture in tube unless otherwise noted)

☑ *Escherichia coli*
☑ *Pseudomonas fluorescens*
☑ *Staphylococcus epidermidis*
☑ *Bacillus megaterium*

MATERIALS

☑ Mueller-Hinton agar plate

☑ sterile swab

☑ antibiotic disks (penicillin, streptomycin, tetracycline, polymyxin B, Trimethoprim sulfamethoxazol, and ciprofloxacin)

☑ forceps or disk dispensers

☑ ruler

PROCEDURE

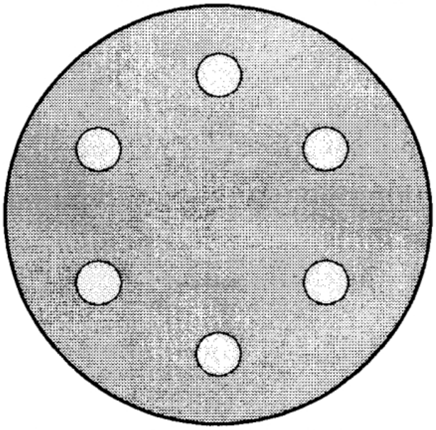
1. Prepare a 3-way swab plate (see Exercise 12.1) on Mueller-Hinton agar for assigned organism(s). Allow to dry.
2. Carefully place each antibiotic disk at intervals on the surface using dispenser and sterile forceps.
3. Incubate plate 18 hours at 37°C.
4. Observe and measure diameter in millimeters of zones of clearing. Determine significance of zone size using Table 18.1.

18.2 Minimal Inhibitory Concentration: E-test

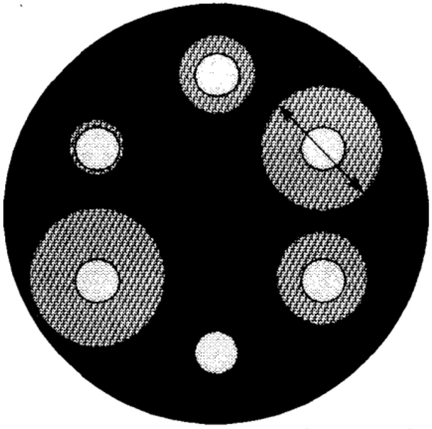
PROCEDURE

1. Prepare a 3-way swab plate (see Exercise 12.1) on Mueller-Hinton agar for assigned organism. Allow to dry.
2. Carefully place the E-test strip (strip must be facing up) on the surface of the medium.
3. Incubate plate 18 hours at 37°C.
4. Read MIC value where the edge of the inhibition ellipse intersects the side of the strip.

FIGURE 18.1 Antibiotic evaluation.



1. Prepare 3-way swab on Mueller-Hinton agar. Place up to six antibiotic disks at intervals. Incubate.



1. Observe growth. Measure the diameter of any zones around disks and compare to antibiotic chart.

FIGURE 18.2 E-test evaluation

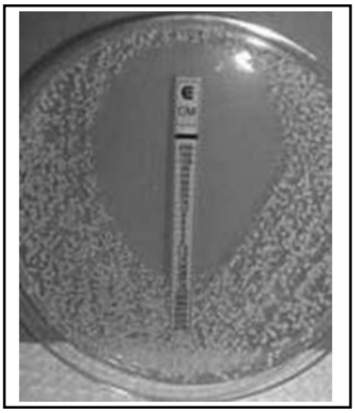


Image courtesy of Roxana Hughes

1. Read MIC value in *u*g/ml. Refer to chart

LABORATORY REPORT 18
ANTIBIOTIC SUSCEPTIBILITY

Name:_______________________________________

RESULTS

18.1 Kirby-Bauer

Measure zone and then use Table 18.1 to determine resistance or sensitivity.

Organism tested: _____________________________

Antibiotic	Zone (mm)	R/I/S
______________	_______	________
______________	_______	________
______________	_______	________
______________	_______	________
______________	_______	________
______________	_______	________

Chart class results:

Organism	Penicillin	Polymyxin B	Streptomycin	Tetracyclin	Ciprofloxacin	Trimethoprim-Sulfamethoxazol
S.epidermidis						
E.coli						
B.megaterium						
P.fluorescens						

18.2 MIC: E-test ™

Antibiotic	MIC(ug/ml)
_____________	__________
_____________	__________

Chart ug/ml results.

Antibiotic	Susceptible	Intermediate	Resistant
Ciprofloxacin	< = 1	2	> = 4
Erythromycin	< = 0.5	1–4	> = 8
Polymyxin	< = 1	2	> = 4

QUESTIONS

1. Identify two modes of action of antibiotics and name an example for each from the antibiotics tested in the experiment: ____

   ____

2. From the class results, which organism was resistant to the most antibiotics tested? Which antibiotic from the experiment would you choose to control this organism? ____________________

   ____

   ____

3. In antibiotic testing, is any zone of clearing always indicative of sensitivity to the antibiotic? Why or why not? ____

   ____

   ____

4. In this experiment, the ____________________ method of antibiotic susceptibility testing was performed on ____________________ agar.

5. Define antibiotic: ____

   ____

   ____

6. Define MIC: ____

   ____

   ____

7. What is the advantage of E-test over Kirby-Bauer sensitivity testing? ____________________

   ____

   ____

8. An agent that kills bacteria is referred to as ______________________________

9. An agent that prevents the growth of bacteria without causing irreversible damage to the bacteria is referred as ____

LABORATORY NINETEEN

Epidemiology

LEARNING OBJECTIVE

Students will be able to:

- Explain how disease is transmitted in a population

PURPOSE

To learn how disease is spread in populations.

INTRODUCTION

Epidemiology is the science that deals with *when* and *where* diseases occur, *how* they are transmitted to the human population and *who* is spreading the disease. People who solve these crucial questions are **epidemiologists**.

When a disease spreads throughout a population of people in a short time it is called an **epidemic**. A **pandemic** is the spread of a given disease worldwide, such as the influenza pandemic which infected millions of people in the 1950s.

The study of the causes and development of disease refers to **pathology** (*pathos*, suffering; *logos*, science). There are many causes of disease. Of course microorganisms make up a large majority of the source of diseases. Other causes include nutritional deficiency (rickets, scurvy, goiter), congenital diseases (German measles, AIDS, fetal alcohol syndrome), genetic disorders (cystic fibrosis, hemophilia, trisomy 21), metabolic diseases (PKU, diabetes mellitus), degenerative diseases (cirrhosis, emphysema), nosocomial (*nosocomium*, hospital; acquired from hospital personnel or equipment), psychogenic (ulcers, colitis, asthma), neoplastic (tumors) and immunological diseases (Lupus, rheumatoid arthritis).

The spread of exotic diseases today from all parts of the world to our own backyards is due to the ease of world travel, mass production and distribution of foods and other goods, person-to-person contact, and **vectors** of different types such as ticks, lice, mosquitoes, and other animals which serve as carriers and disseminators for bacteria.

There are many ways by which disease spreads. It can be done by person-to-person contact as in sexually transmitted diseases (STDs such as gonorrhea, syphilis, herpes, *Chlamydia*) or it can simply be spread by infected hands. The contaminated hands of health care providers is a major cause of spreading disease microorganisms in hospitals.

The Center for Disease Control and Prevention (CDC) in Atlanta, Georgia is an agency of the Department of Health and Human Services, Public Health Service. It is the duty of the scientists and epidemiologists at the CDC to track trends and analyze new and reemerging infectious disease issues around the world. (Their journal published by the National Center for Infectious Diseases, *Emerging Infectious Diseases*, may be accessed at www.cdc.gov or www.cdc.gov/ncidod/EID/eid.htm.)

In this laboratory exercise we will discover the ease and rapidity of the spread of a common "pathogen" throughout a small population represented by the members of this lab. The ease of disease dissemination through a simple handshake will be shown. Each person in the class will infect her or his glove with normal flora—that is, except for one person who will be spreading the "dummy pathogen" to the rest of the population. After two rounds of handshaking, it will be determined how many people have been infected by the "diseased" person.

LABORATORY EXERCISE

19.1 Mock Epidemic.

ORGANISMS

(liquid culture in tube unless otherwise noted)

☑ *Micrococcus luteus*

☑ *Serratia marcescens*

MATERIALS

☑ sterile Petri dish containing a Durham (6 mm x 50 mm glass) tube

☑ disposable gloves

☑ sterile cotton swabs

☑ inoculating loop

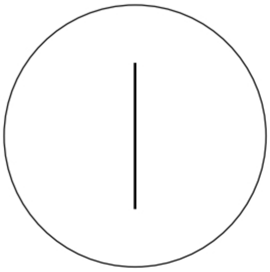
☑ 2 Tryptic Soy Agar (TSA) plates

PROCEDURE

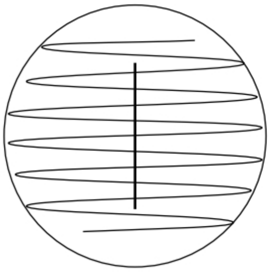
Just prior to lab, the teaching assistant (TA) will add 2 ml of *Micrococcus luteus* culture to the sterile Petri dishes for all but one of the students. In the remaining Petri dish, 2 ml of *Serratia marcescens* will be added. The plates will be numbered and distributed randomly to the students.

1. Each student is presented with a contaminated Durham tube in a numbered Petri dish. One of the tubes has been contaminated with *Serratia marcescens* (red pigment, represents the "disease"). The rest with *Micrococcus luteus* (yellow pigment, represents "normal microbiota"). After reading the entire experiment and listening to instructions from the TA, each student should obtain one glove, one rich plate and one sterile swab (those students who have sample numbers 5, 10, 15, 20, or 24 will need two plates and 2 swabs). Students should each label their plates with their sample # and, if they have two sets, label "Round 1" and "Round 2".
2. Each student puts a glove on the right hand. On a signal from the TA, open the plate and rubs the contaminated tube on the palm of the gloved hand. The tube is returned to the Petri dish.

3. Again on a signal from the TA, the student with Petri dish #1 shakes hands with any other student (NOTE: All handshakes should be palm to palm). The student with Petri dish #2 does likewise with any student. This continues in sequence until each person has shaken hands with a person of choice. The TA records on the blackboard the sequence of handshakes.
4. Students with Petri dish numbers 5, 10, 15, 20, and 24 swab their right palm with a sterile cotton swab. Using this swab, make a single streak in the center of a TSA plate which is labeled with sample # and “1st Round”.



5. When step 4 complete, a second round of handshakes is again initiated by the student with Petri dish #1. Each student should shake hands with a different student from the one they chose in the first round. The TA again records the sequence of handshakes.
6. When the second round is completed, all students rub their right palms with a sterile swab and swab the agar plate provided. Students with Petri plate numbers 5, 10, 15, 20, and 24 should use fresh swabs and fresh TSA plates which are labeled with sample # and “2nd Round”.
7. After swabs are completed, dispose of gloves and used swabs in biohazard bag.
8. Finally, use a sterile loop to complete streaking each plate using the pattern below. Incubate plates at 25°C for 2–5 days.



9. Observe growth on the plates. Students with only yellow colonies are “uninfected”, while those with any red colonies have contracted the “disease”. Collect the status of each student’s plates on the results chart. Try to determine who began the epidemic by tracing back to the number of the originally infected plate.

LABORATORY REPORT 19
EPIDEMIOLOGY

Name:_______________________________________

RESULTS

Fill in the chart below with your class handshake information and results. From this information, determine the number of the person most likely to have begun the epidemic: _______________.

TABLE 25.1 Epidemiology results.

Student Number	Handshake Round 1	Handshake Round 2	Results Round 1	Results Round 2
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				

QUESTIONS

1. What was the purpose of the Epidemiology lab ____

2. Define "communicable diseases" ____

3. Describe an "epidemiologist" ____

4. An epidemic that becomes global is referred to as ____

5. What is the purpose of the Center for Disease Control and Prevention?

SECTION FIVE

Survey of Microorganisms

Since 1980 or so it has been believed that there are three major groups of life on Earth. This three "domain" system was spearheaded by Carl Woese, Ralph Wolfe, Norman Pace and colleagues at the University of Illinois, when they discovered in 1977 a new domain, namely Archaea, which contained the Archaebacteria. Thus prokaryotes are found in two of the three domains as Eubacteria and Archaea while eukaryotes are all in one domain of Eukarya. In the following exercises we will examine the prokaryotes containing bacteria and archaea, and we will examine such eukaryotes as algae, protozoa and fungi (including yeasts and molds).

LABORATORY TWENTY

Fungi and Lichens

LEARNING OBJECTIVE

Students will be able to:

- Survey the diversity of fungi and similar organisms

PURPOSE

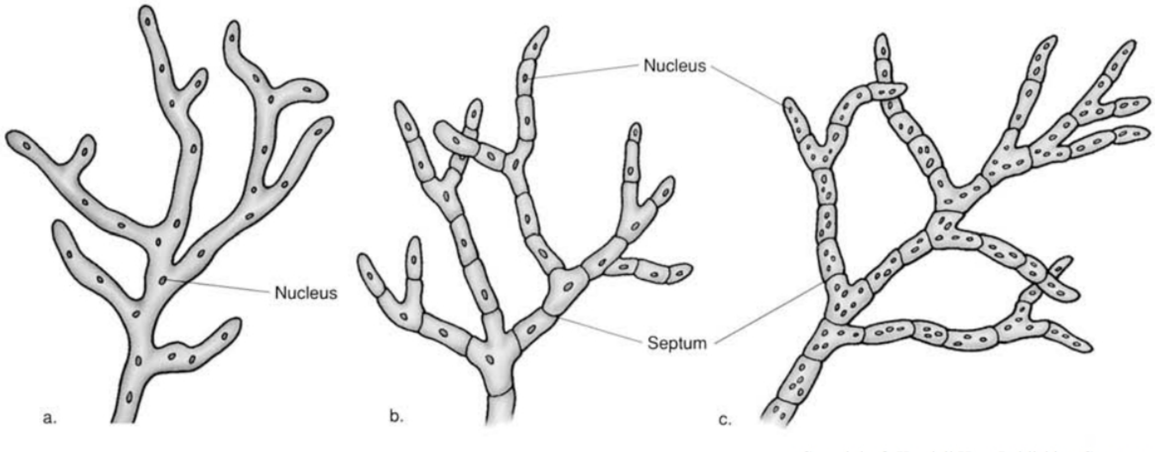
To identify microorganisms belonging to the fungi, lichens, and filamentous bacteria.

INTRODUCTION

Fungi encompass a large group of chemoheterotrophic eukaryotes that must consume preformed organic matter. This group includes yeasts and molds, slime molds, water molds, mushrooms, puffballs and bracket fungi. The fungi can live as saprophytes digesting and consuming dead plants and animals or they may live as parasites assimilating tissues of living plants and animals. The study of fungi is called mycology.

Some fungi such as yeasts are unicellular but most are multicellular, producing vegetative or reproductive structures. **Hyphae** (singular: **hypha**) consist of a series of elongated tubular cells. Cross-walls (**septa**) occur within hyphae, dividing them into separate cells (**septate**). Some hyphae are non-septate. A mass of hyphae is known as a **mycelium** which supports the nutrition

FIGURE 20.1 Type of hyphae found in fungi.



Copyright © Kendall Hunt Publishing Company

of the organisms. The whole body of the fungus is called a **thallus**. The cells in the hypha can be uninucleate (one nucleus, septate), binucleate or multinucleate (non-septate). Non-septate, multinucleate cells are said to be coenocytic meaning many nuclei in a common cytoplasm.

All fungi excrete digestive enzymes to degrade and solubilize food molecules so that they can be taken up by the fungal cell. Fungi are surrounded by a rigid cell wall, 0.2 μm thick, that is made mostly of chitin, a polymer of N-acetyl glucosamine. The wall also contains cellulose, proteins and some lipids. As eukaryotes, fungi contain a true nucleus, nucleolus, nuclear membrane, mitochondria and mitochondrial membrane, vacuoles and Golgi apparatus. The nucleus contains a diploid set of linear chromosomes coated with histones while the mitochondrion has a circular bacterial-type chromosome in addition to several plasmids.

The fungi can be divided into three major groups that reproduce by asexual and sexual means. Sexual reproduction involves the union of two haploid cells. In the fungi, sexual reproduction can involve undifferentiated hyphae, gametes or gametangia.

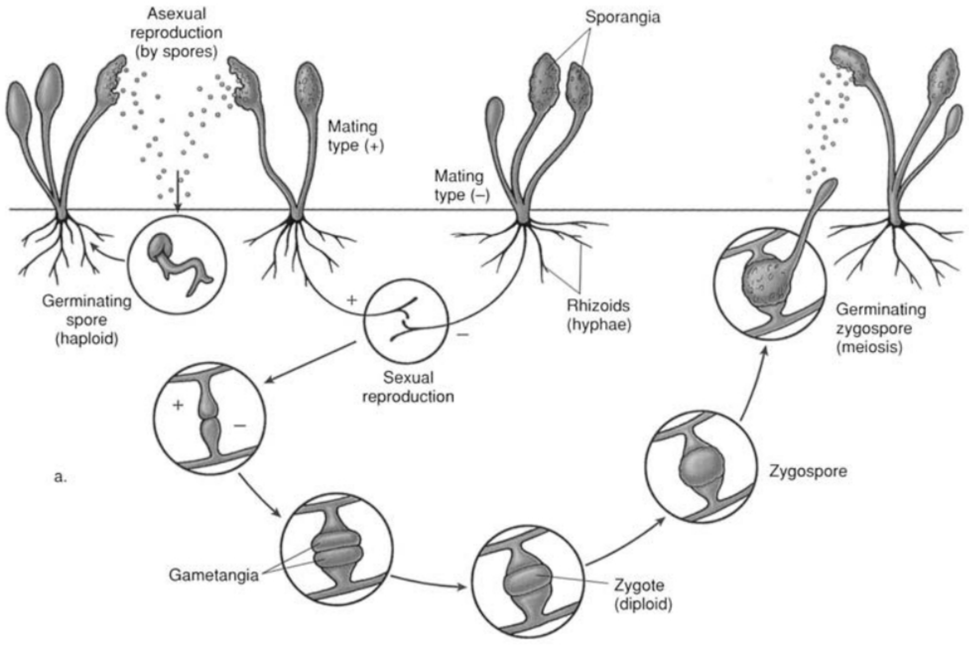
In summary, fungi are non-motile, non-photosynthetic and chemoheterotrophic. Asexual reproduction is by budding, conidiospores, arthrospores, chlamydospores, sporangiospores, oospores and in one genus, transverse fission.

The three major groups of fungi are:

I. Zygomycota or Zygomycetes

1. Mycelia and hyphae have no cross-walls (non-septate).
2. Coenocytic multinucleate.
3. Sexual reproduction is by **ZYGOspores**, after which the group is named. Two gametangia (one + mating type, the other -) fuse to form a zygote which then forms a thick-walled zygospore (e.g. *Mucor*).
4. Non-sexual reproduction is by many **sporangiospores** produced in a sporangium or purse.
5. Examples of group are *Mucor, Rhizopus, Cunninghamella, Phycomyces*

FIGURE 20.2 Zygomycota life cycle.



Copyright © Kendall Hunt Publishing Company

II. Ascomycota or Ascomycetes

2000 genera in this group. Include yeasts, powdery mildews, cup fungi and the edible delicacies, the morels and truffles. Members of the group are mostly terrestrial with a few fresh water or marine members. Plant diseases include Dutch elm disease, chestnut blight, and blackspot of rose. Yeasts are important for alcohol and bread while many fungi have medicinal significance.

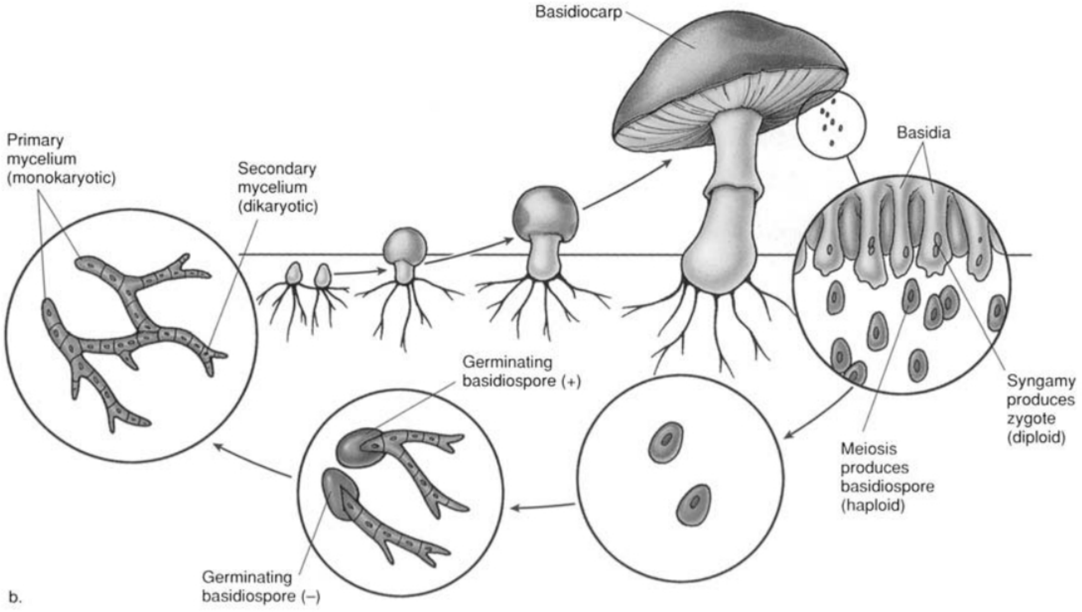
1. Mycelium and hyphae have cross-walls (septate).
2. Sexual reproduction is by sexual **ASCOspores** formed within an **ascus** which gives the group its name.
3. Non-sexual reproduction is by **conidiospores** in a **conidium**, by **oidia** where the mycelium fragments, by budding (*Saccharomyces*) and by binary fission (*Schizosaccharomyces*, a fission yeast).
4. Examples of group: *Saccharomyces*, *Claviceps*, *Schizosaccharomyces*, *Neurospora*, *Sordaria*, *Taphrina*, *Morchella*

III. Basidiomycota or Basidiomycetes

Group contains about 1,589 genera and more than 31,000 species and includes jelly fungi, rusts, smuts, bracket fungi, mushrooms and puffballs.

1. Mycelium and hyphae have cross walls (septate).
2. Sexual reproduction is by **BASIDIOspores** produced in a **basidium** which gives the group its name. Basidia are organs homologous to the ascus.
3. Non-sexual reproduction is by buds, conidia, chlamydospores and oidia.
4. Examples of group: *Amanita* (poisonous mushroom), *Agaricus* (field mushroom), *Coprinus* (inky cap mushroom), *Puccinia* (rust), *Cyathus striatus* (bird's nest fungus), *Phanerochaete chrysosporium* (white rot fungus)

FIGURE 20.3 Basidiomycota life cycle.


Basidiocarp
Primary mycelium (monokaryotic)
Secondary mycelium (dikaryotic)
Basidia
Germinating basidiospore (+)
Syngamy produces zygote (diploid)
Meiosis produces basidiospore (haploid)
Germinating basidiospore (–)
b.

Copyright © Kendall Hunt Publishing Company

Dimorphic fungi. Many fungi are dimorphic, especially those which cause diseases in animals and humans. They have two forms. They can change from the yeast (Y) form which is pathogenic in animals to the mold or mycelial form (M) in the environment. This form is saprophytic. This Y-M shift occurs in response to temperature or nutrition changes, and is important in demonstrating pathogenicity in clinical identification (Example: *Candida albicans*).

Lichens. Lichens are the result of a mutualistic association (both partners benefit) between a fungus, usually an Ascomycete and a phototroph, a green alga or a cyanobacterium. The two partners function so well as a unit that more than 15,000 species of lichen have been described. The fungus is the dominant partner, making up most of the mass and gives the thallus its structure. Fungal mycelia extend below the lichen to form rhizoids which allow the lichen to attach to its substrate. The algal partner usually grows as individual cells in the lichen and provides carbohydrates from photosynthesis. When a cyanobacterium is the photosynthetic partner the nitrogen requirement must also be supplied in addition to carbohydrates.

Blocked due to copyright restrictions.

Blocked due to copyright restrictions.

LABORATORY EXERCISE

ORGANISMS

PLATE CULTURES

(single central inoculation unless otherwise noted)

☑ *Aspergillus*

☑ *Fusarium*

☑ *Penicillium*

☑ *Rhizopus*

☑ *Saccharomyces cerevisiae* (streaked for isolation)

LIQUID CULTURES

☑ *Saccharomyces cerevisiae*

☑ *Fusarium* spp

PREPARED SLIDES

☑ *Rhizopus* zygospore

☑ *Sordaria* perithiceum

☑ *Peziza* ascospore

☑ *Coprinus* basidiospores

OTHER

☑ Lichen sample on rock or branch

20.1 Environmental Sampling of Fungi.

NOTE: Additional fungal samples may be obtained if each student exposes a plate of Sabouraud dextrose agar to the air either indoors or outdoors for 15 minutes a week prior to this exercise. These plates should be incubated upright at 30°C.

SUPPLIES

☑ Linder's cotton blue

☑ Clear cellophane tape

☑ microscope slides

☑ coverslips

☑ Pasteur pipettes and bulbs

PROCEDURE

1. Observe the macroscopic appearance of each of the provided plate cultures and record on laboratory report. Note similarities and differences between the filamentous fungi, yeast colonies, and filamentous bacteria.
2. Prepare a modified wet mount to microscopically examine the plate cultures. Using a piece of clear cellophane tape held at both ends to form a loop with the adhesive side out, gently press the surface of the growth and then remove. Some fungal material will adhere to the tape. Place the piece of tape containing specimen into a small drop of Linder's cotton blue on a microscope slide. The tape will serve the same purpose as a coverslip while the adhesive preserves structures present in the specimen. Observe at 100X and 400X magnification. Sketch any spore-forming structures present in each sample.
3. Prepare wet mounts of the liquid cultures. Prepare these by placing a drop of Linder's cotton blue on the slide and adding one loopful of the culture to be examined. Place a coverslip over the drop as described in Exercise 1.1.

LABORATORY REPORT 20
FUNGI, LICHENS AND FILAMENTOUS BACTERIA

Name:______________________________________

RESULTS

Sketch below and identify the genus of each organism observed microscopically. Note also macroscopic properties and any microscopic features which are helpful in distinguishing each.

Plates

Aspergillus

Fusarium

Penicillium

Rhizopus

Slides

Rhizopus
zygospores

Peziza
ascospores

Coprinus
basidiospores

Sordaria
perithiceum

Wet mount

S. cerevisiae

Fusarium spp

QUESTIONS

1. How are the fungi classified (by what characteristic)? ____________________________________

   Name the three groups: ________________________

   ____

   ____

2. Compare at least two properties that differ between the fungi and the filamentous bacteria:

   Fungi:

   1) ___________________

   2) ___________________

   Bacteria:

   1) ___________________

   2) ___________________

3. What is the unicellular form of fungi called? ________________

   Give the genus of one example studied in this laboratory which exists in the unicellular form?

   ____

4. What media was used in this laboratory for growing fungi? ____________________________________

   What properties make this media useful for growing fungi? ____________________________________

   ____

5. The Fungi belong to the Domain ________________

6. What is the difference between yeast and molds?

LABORATORY TWENTY-ONE

Cyanobacteria, Algae, and Protozoa

LEARNING OBJECTIVE

Students will be able to:

- Identify and name representative species of cyanobacteria, algae and protozoa

PURPOSE

To survey the diversity of the cyanobacteria, algae, and protozoa.

INTRODUCTION

Cyanobacteria.

The **cyanobacteria** are photosynthetic prokaryotic cells that carry out oxygenic photosynthesis like plants and algae. Cyanobacteria (Figure 21.1) contain **chlorophyll *a*,** which is not present in other prokaryotes. In addition to chlorophyll a, these bacteria contain **ß-carotene, xanthophylls,** blue **phycocyanin** and red **phycoerythrin.** The cyanobacteria have photosynthetic sac-like membranes known as **thylakoids** resembling those of plants and algae. Many cyanobacteria fix nitrogen in specialized cells called **heterocysts** (Fig. 21.1). *Anabaena, Oscillatoria, Gleocapsa, Spinulina, Anacystis, Agmenellum, Prochloron, Chroococcus* and *Nostoc* are well known members.

Algae.

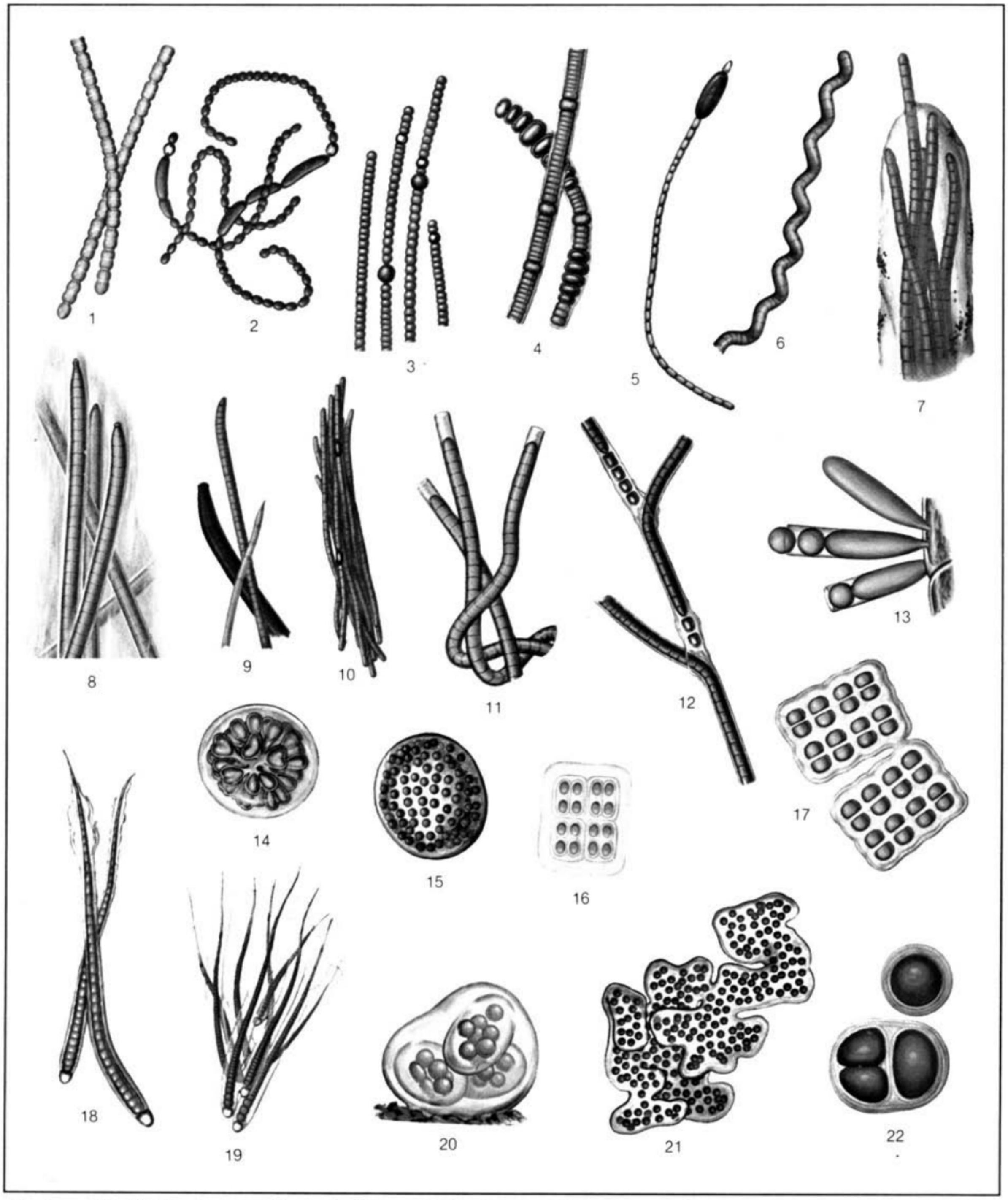
Algae are aerobic eukaryotes which carry out plant photosynthesis. They may be unicellular, or multicellular with no significant tissue differentiation. They are quite often motile and may have a cellulose wall. Algae are common inhabitants of ponds, lakes, along ocean shorelines, and in the *surface* water of the oceans. Since water absorbs light, they do not grow at depths greater than a few hundred feet.

Separation of the algae into groups is based on (1) the pigments found in their plastids (2) food reserves, and (3) cell walls. The study of algae is called phycology.

Algae may be unicellular (Fig. 21.2, #1-6), colonial (Fig. 21.2, #13, 14, 15, 19, 20), filamentous, and multicellular (Fig. 21.3). A few are differentiated and bladelike, and a few produce primitive roots, stems and blades.

Green Algae (e.g. *Volvox*, *Ulva*; contain: ß-carotene, xanthophylls). Also known as Chlorophycophyta. Green algae contain chlorophyll a and b and photosynthetic pigments. They store

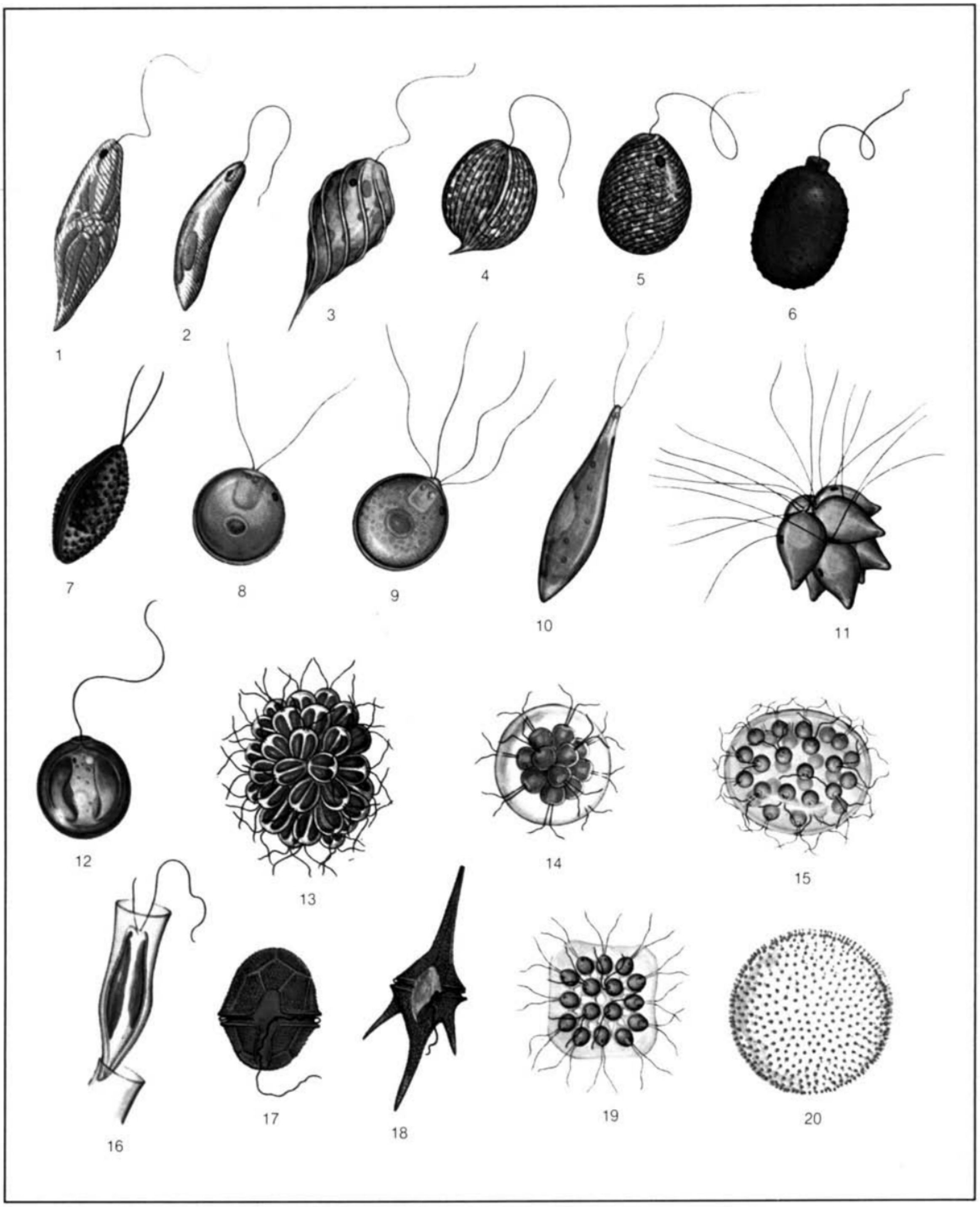
FIGURE 21.1 Cyanobacteria



Courtesy of the U.S. Environmental Protection Agency, Office of Research & Development, Cincinnati, Ohio 45268.

1. *Anabaena* (350X)
2. *Anabaena* (350X)
3. *Anabaena* (175X)
4. *Nodularia* (350X)
5. *Cylindrospermum* (175X)
6. *Arthrospira* (700X)
7. *Microcoleus* (350X)
8. *Phormidium* (350X)
9. *Oscillatoria* (175X)
10. *Aphanizomenon* (175X)
11. *Lyngbya* (700X)
12. *Tolypothrix* (350X)
13. *Entophysalis* (1000X)
14. *Gomphosphaeria* (1000X)
15. *Gomphosphaeria* (350X)
16. *Agmenellum* (700X)
17. *Agmenellum* (175X)
18. *Calothrix* (350X)
19. *Rivularia* (175X)
20. *Anacystis* (700X)
21. *Anacystis* (175X)
22. *Anacystis* (700X)

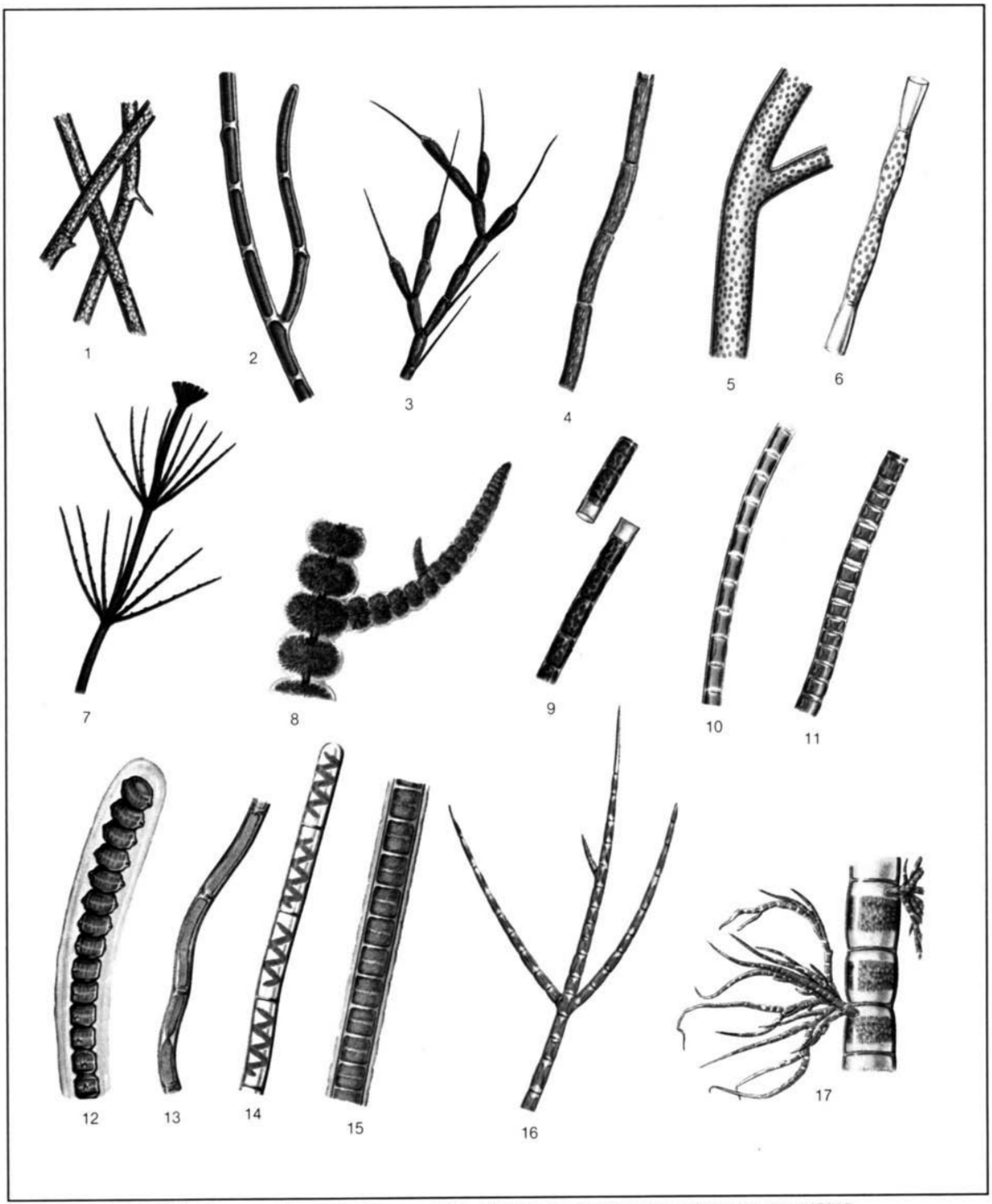
FIGURE 21.2 Flagellated algae.



Courtesy of the U.S. Environmental Protection Agency, Office of Research & Development, Cincinnati, Ohio 45268.

1. *Euglena* (700X)
2. *Euglena* (700X)
3. *Phacus* (1000X)
4. *Phacus* (350X)
5. *Lepocinclis* (350X)
6. *Trachelomonas* (1000X)
7. *Phacotus* (1500X)
8. *Chlamydomonas* (1000X)
9. *Carteria* (1500X)
10. *Chlorogonium* (1000X)
11. *Pyrobotrys* (1000X)
12. *Chrysococcus* (3000X)
13. *Synura* (350X)
14. *Pandorina* (350X)
15. *Eudorina* (175X)
16. *Dinobyron* (1000X)
17. *Peridinium* (350X)
18. *Ceratium* (175X)
19. *Gonium* (350X)
20. *Volvox* (100X)

FIGURE 21.3 Filamentous algae.



Courtesy of the U.S. Environmental Protection Agency, Office of Research & Development, Cincinnati, Ohio 45268.

1. *Rhizoclonium* (175X)
2. *Cladophora* (100X)
3. *Bulbochaete* (100X)
4. *Oedogonium* (350X)
5. *Vaucheria* (100X)
6. *Tribonema* (300X)
7. *Chara* (3 X)
8. *Batrachospermum* (2 X)
9. *Microspora* (175X)
10. *Ulothrix* (175X)
11. *Ulothrix* (175X)
12. *Desmidium* (175X)
13. *Mougeotia* (175X)
14. *Spirogyra* (175X)
15. *Zygnema* (175X)
16. *Stigeoclonium* (300X)
17. *Draparnaldia* (100X)

starch and oil and have cellulose in their cell walls. Unicellular members of the group include *Pleurococcus*, *Chlorella*, and *Chlamydomonas* (Fig. 21.2 and 21.4). In most cases, a sexual life cycle is common, with motile isogametes. Filamentous members of the phylum include *Ulothrix, Spirogyra*, and *Cladophora* (Fig. 21.3).

Red Algae A.k.a. Rodophyta. Red algae have red plastids and contain chlorophyll *a* and phycobilins as photosynthetic pigments. They store Floridean starch (like glycogen) or cellulose with agar and carrageenan in their cell walls. The red algae are of great importance to microbiologists because they are the commercial source of **agar**. Red algae produce carrageenan which is a major food source in the Orient.

Golden Algae A.k.a. Chrysophyta. Plastids are yellow, yellow-green or golden-brown. They contain chlorophyll *a,* c_1/c_2 pigments. They store starchlike carbohydrates and oils. Many unicellular species including Diatoms (see below). Filamentous golden algae include *Vaucheria* (Fig. 21.3, #5) their cell surface is covered by characteristic scales.

Diatoms (Fig. 21.5). A.k.a. Bacillariophyta. They store polysaccharides and oils and contain silicon embedded in a pectin matrix in their cell walls. Diatoms are highly resistant to degradation and contain the best microfossils. Accumulated silicon shells (cell wall) of diatoms form “diatomaceous earth“ so prominent in the world’s largest diatom quarry in Lompoc, California. Diatomaceous earth is used as a filter to clarify cloudy wines, fruit juices, cane sugar and other beverages, as an insulator in steam boilers, as an abrasive in toothpaste and cosmetics. Diatoms are very beautiful with exquisite shapes. They are found in cold waters being the most plentiful form of phytoplankton in the Arctic. They provide an ever-present food supply for aquatic animals.

Brown Algae. A.k.a. Phaeophyta. Contain chlorophyll *a* and *c* and xanthophylls such as fucoxanthin. They store laminarin, fats and mannitol, a sugar alcohol. They contain cellulose and alginic acid (or algin) in their cell walls with two lateral unequal flagella. Multicellular. The brown algae are found in salt water, attached to rocks and on the ocean shores. They vary in size from microscopic plants to 200 feet in length. The giant kelp, *Macrocystis pyrifera*, and *Fucus*, a seaweed, belong to this group.

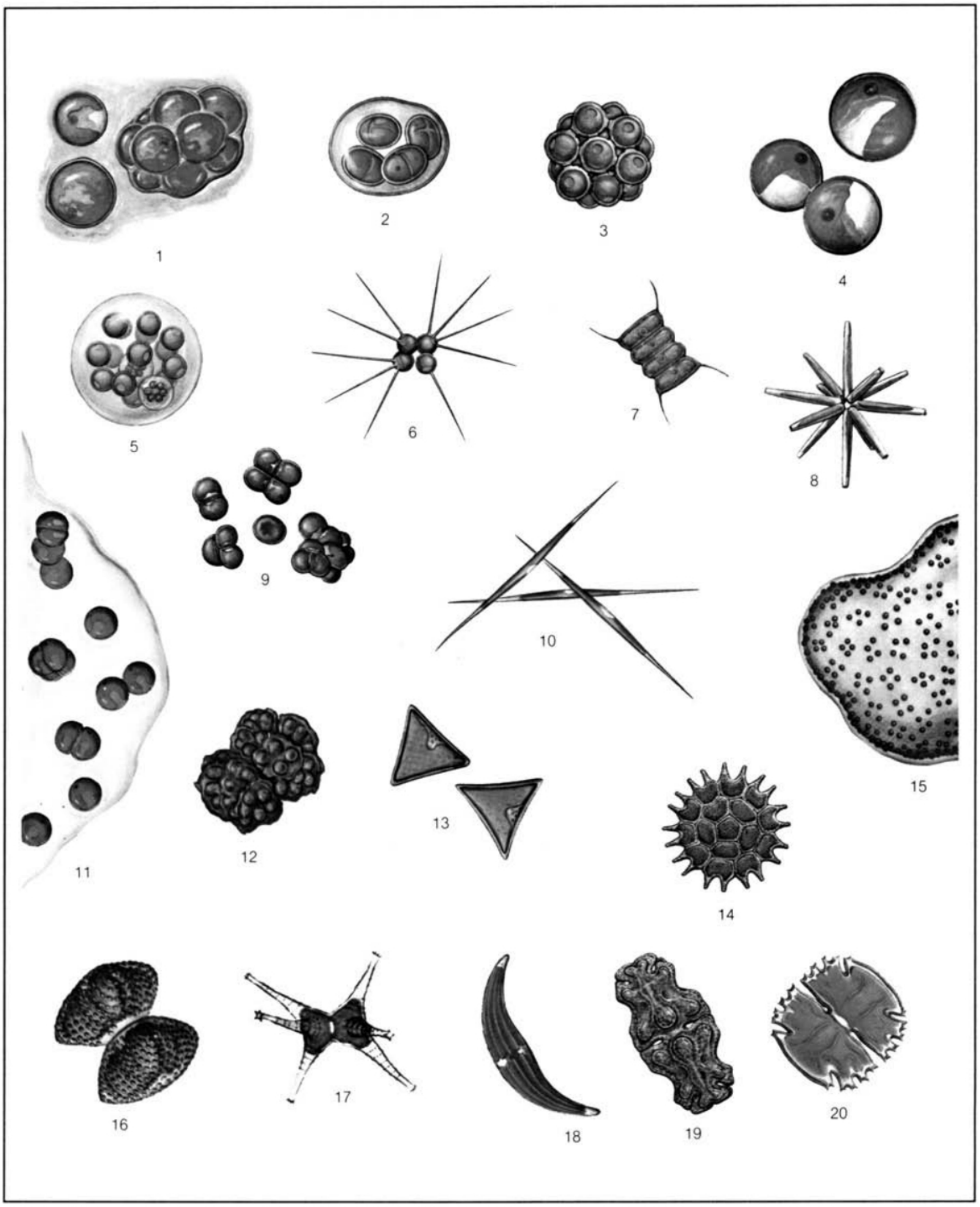
Dinoflagellates A.k.a. Pyrrophyta. They store starch and oils with cellulose cell walls. They are red and abundant in tropical water. Like diatoms, they are important components of the phytoplankton. They cause “red tides“ or “blooms“ in warm coastal waters which kill fishes. They excrete a powerful neurotoxin, and they are toxic to humans who eat shellfish fed on the algae. The nerve toxin is not toxic for the shellfish! The red tides have been seen in the Gulf of Mexico, San Francisco (seen as you fly out of the SF airport), the Pacific North-West and recently New England. Reproduce by asexual cell division.

Protozoa.

Protozoa are non-photosynthetic, unicellular eukaryotes (Fig. 21.6). They are known to cause devastating human diseases including malaria (infection of RBCs), leishmaniasis (infection of WBCs) and African sleeping sickness (infection of the nervous system).

Protozoa are the champions when it comes to unicellular differentiation. They have elaborate organelles such as a **cytosome** (mouth), **cilia** (sweep food into mouth; move cell), **food vacuoles** (digest food), **cytoproct** (anus-like structure through which undigested food expelled), **contractile vacuole** (osmotic pressure regulator), **micronucleus** (2N nucleus required for meiosis), **macronucleus** (polyploid with many sets of chromosomes) and a **pellicle** (stiff layer of protein fibers or calcium-like or silica-like structures).

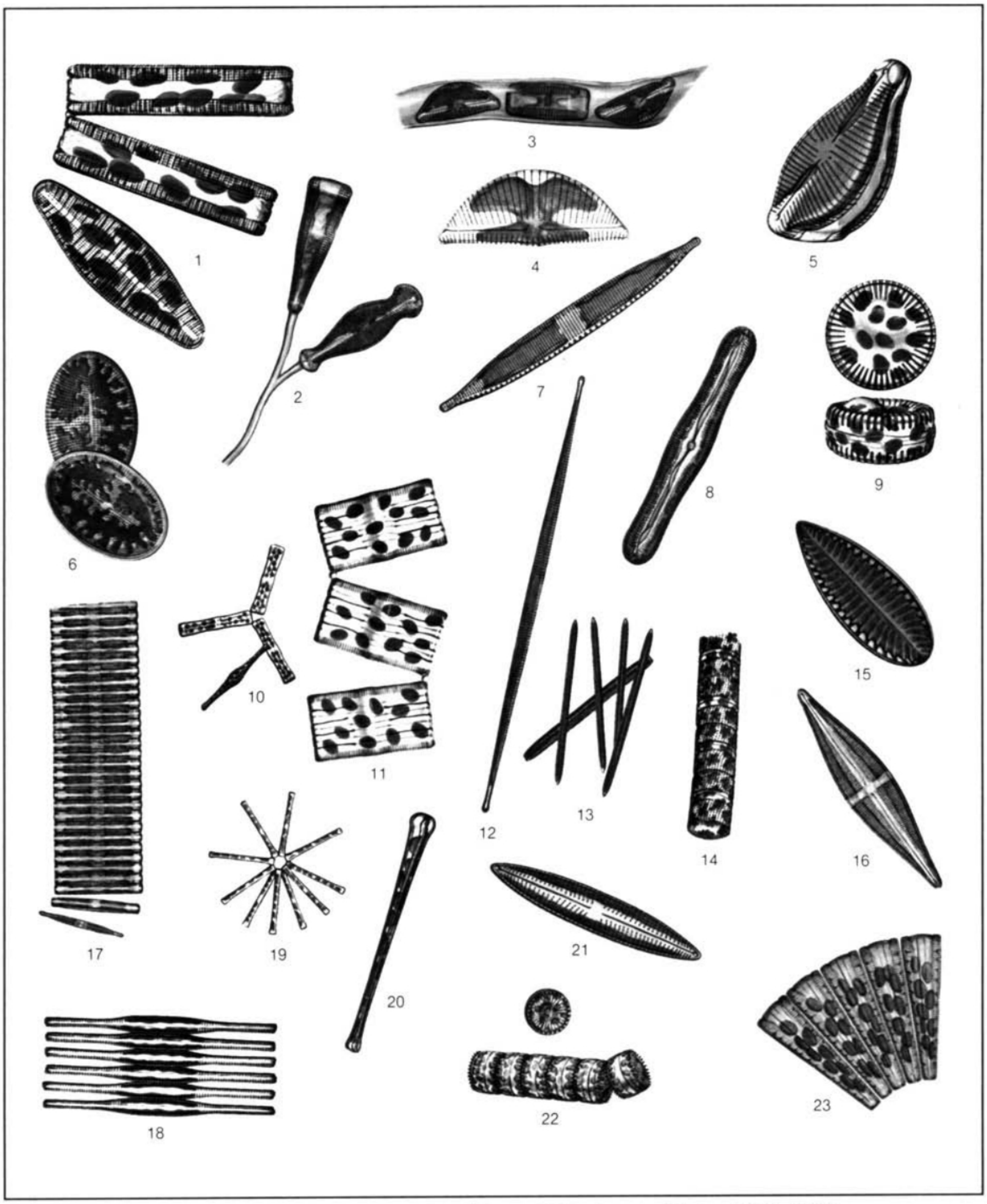
FIGURE 21.4 Nonfilamentous and nonflagellated algae.



Courtesy of the U.S. Environmental Protection Agency, Office of Research & Development, Cincinnati, Ohio 45268.

1. *Chlorococcum* (700X)
2. *Oocystis* (700X)
3. *Coelastrum* (350X)
4. *Chlorella* (350X)
5. *Sphaerocystis* (350X)
6. *Micractinium* (700X)
7. *Scendesmus* (700X)
8. *Actinastrum* (700X)
9. *Phytoconis* (700X)
10. *Ankistrodesmus* (700X)
11. *Pamella* (700X)
12. *Botryococcus* (700X)
13. *Tetraedron* (1000X)
14. *Pediastrum* (100X)
15. *Tetraspora* (100X)
16. *Staurastrum* (700X)
17. *Staurastrum* (350X)
18. *Closterium* (175X)
19. *Euastrum* (350X)
20. *Micrasterias* (175X)

FIGURE 21.5 Diatoms.



Courtesy of the U.S. Environmental Protection Agency, Office of Research & Development, Cincinnati, Ohio 45268.

1. *Diatoma* (1000X)
2. *Gomphonema* (175X)
3. *Cymbella* (175X)
4. *Cymbella* (1000X)
5. *Gomphonema* (2000X)
6. *Cocconeis* (750X)
7. *Nitschia* (1500X)
8. *Pinnularia* (175X)
9. *Cyclotella* (1000X)
10. *Tabellaria* (175X)
11. *Tabellaria* (1000X)
12. *Synedra* (350X)
13. *Synedra* (175X)
14. *Melosira* (750X)
15. *Surirella* (350X)
16. *Stauroneis* (350X)
17. *Fragillaria* (750X)
18. *Fragillaria* (750X)
19. *Asterionella* (175X)
20. *Asterionella* (750X)
21. *Navicula* (750X)
22. *Stephanodiscus* (750X)
23. *Meridion* (750X)

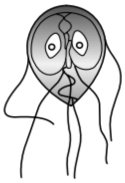
Blocked due to copyright restrictions.

Protozoa reproduce asexually by fission and budding. Some protozoa, notably *Plasmodium*, reproduce by schizogony or multiple fission. Each nucleus divides repeatedly and then some cytoplasm gathers around each nucleus to form daughter cells. At times, a giant cell undergoes many fissions without growth, producing many small cells. Sexual reproduction is by **conjugation**, the fusion of two vegetative cells or by fusion of two specialized cells, called gametocytes.

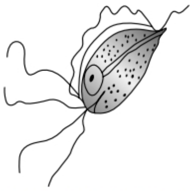
During their life cycle, some protozoa produce **cysts** which are highly resistant, non-growing units. Polysaccharide capsules surround the cysts.

Protozoa have been historically classified into four groups based on their motility. This is not a phylogenetic based scheme.

I. The flagellate protozoa or **Mastigophorans** move by flagella, most have two flagella, and they are covered by an expanded membrane called an undulating membrane that propels the cell by undulating as the flagellum inside moves. There are 2 subgroups, the Archaeozoa (flagellate with no mitochondria) and Euglenozoa (flagellates with mitochondria).



Giardia trophozoite



Trichomonas vaginalis

Members include *Trypanosoma cruzi* (Chaga's disease), *Giardia lamblia* (dysentery), *Trichomonas vaginalis* (vaginal and urethral infection), *Euglena acus* that have chloroplast and was previously classified among the algae

II. The amoeboid protozoa or **Sarcodinas** move by pseudopodia (false feet). They are found in marine and fresh waters and in the soil. They are a huge group with all members having the ability to produce pseudopodia at some stage of the life cycle. Pseudopods are organelles of locomotion and feeding. Members include *Entamoeba, Amoeba, Dientamoeba, Iodamoeba, Endolimax* (all causing amoeboid dysentery), and *Naegleria fowleri* (a rare fatal form of encephalitis from swimming in infected waters). *Dientamoeba fragilis* is found in the human intestine in 4% of humans. It causes a mild diarrhea.

III. The ciliate protozoa or **Ciliophora** move by cilia. Most have two nuclei, the **micronucleus** (necessary only for sexual reproduction) is diploid, and the **macronucleus** is polyploid (several copies of its complement of chromosomes) and directs vegetative growth and cell division. Cells without the micronucleus grow normally but reproduce only asexually.

Ciliates like most protozoa prey on bacteria, fungi and other protozoa. Predation is very specific. For example, *Didinium nasatum* attacks only another species of *Paramecium* which it swallows whole. This swells its cytosome greatly which prevents the little cannibal from eating for another two hours (a lifetime for a protozoan!).

Many ciliates are associated with animals. The rumen of cows and other chewers-of-the-cud abound with ciliates that digest cellulose for the animal. Only one ciliate, *Balantidium coli*, is a human pathogen (a severe diarrhea infection of the colon).

Members include *Paramecium, Vorticella, Stentor, Tetrahymena, Didinium, Balantidium*, and *Blepharisma*.

IV. The **Sporozoa** are non-motile. All are parasitic so there is less need to move. This group has elaborate life cycles where they exist as different stages. A **trophozoite** is the active multiplying vegetative stage. When it is infectious to the animal or human host it is called a **sporozoite** and when it enters and infects RBCs it is a **merozoite**. Sometimes as in malaria (*Plasmodium*) more than one host is involved, human and mosquito (*Anopheles*). Typically, in the life cycle, schizogony of the merozoites occurs causing the characteristic sudden attacks of shaking chills and high fever in malaria.

Members include *Plasmodium (P. vivax, P. falciparum)*, and *Toxoplasma gondii* (found in cats, dogs, cattle, sheep and humans). Humans get the disease (systemic, toxoplasmosis) from eating cysts in infected meat or from contamination by cat feces.

LABORATORY EXERCISE

For each of the sections below, observe the organisms using wet mounts (see Exercise 1.1).

MATERIALS

☑ Transfer pipettes
☑ microscope slides
☑ cover slips
☑ lens paper

21.1 Cyanobacteria.

ORGANISMS

(liquid culture in tube unless otherwise noted)

☑ *Anabaena*
☑ *Gleocapsa*
☑ *Spirulina*
☑ *Oscillatoria*

21.2 Algae.

ORGANISMS

(liquid culture in tube unless otherwise noted)

☑ *Chlamydomonas*
☑ Desmid/Diatom mix
☑ *Volvox*
☑ *Spirogyra*
☑ Other samples as assigned

21.3 Protozoa.

Blocked due to copyright restrictions.

ORGANISMS

(liquid culture in tube unless otherwise noted)

☑ *Amoeba proteus*
☑ *Blepharisma*
☑ *Paramecium caudatum*
☑ *Vorticella*
☑ *Euglena acus*
☑ Other samples as assigned

PREPARED SLIDES:

☑ *Plasmodium*
☑ *Trypanosoma* spp.
☑ *Giardia lamblia*
☑ *Trichomonas vaginalis*

LABORATORY REPORT 21
CYANOBACTERIA, ALGAE AND PROTOZOA

Name:______________________________________

RESULTS

Sketch below and identify the genus of each organism observed in the laboratory. Note any microscopic features which are helpful in distinguishing each.

Cyanobacteria

Algae

Protozoa

QUESTIONS

1. Name two of the characteristics which can be used to classify algae? ______________________________

____

2. Protozoans are classified based on their mode of ______________________.

3. Which contains heterocysts, algae or cyanobacteria? ___________________

What is this structure and what is its function? ______________________________

____

4. Of the organisms studied in this laboratory, two groups contain membrane-bound nuclei and mitochondria, making them eukaryotic. Name these two groups: ______________________________

____

Name the prokaryotic group: _____________________________________

SECTION SIX

Environmental Microbiology

LABORATORY TWENTY-TWO

Water Analysis Membrane Filtration, Colisure Test, MPN

LEARNING OBJECTIVES

Students will be able to:

- Use a membrane filter system to identify and count coliforms in a water sample.
- Identify coliforms and *Escherichia coli* using the Colisure system.
- Perform the most probable number method for coliform detection in a sample.

PURPOSE

This laboratory will introduce students to techniques utilized to detect sewage contamination in water sources.

INTRODUCTION

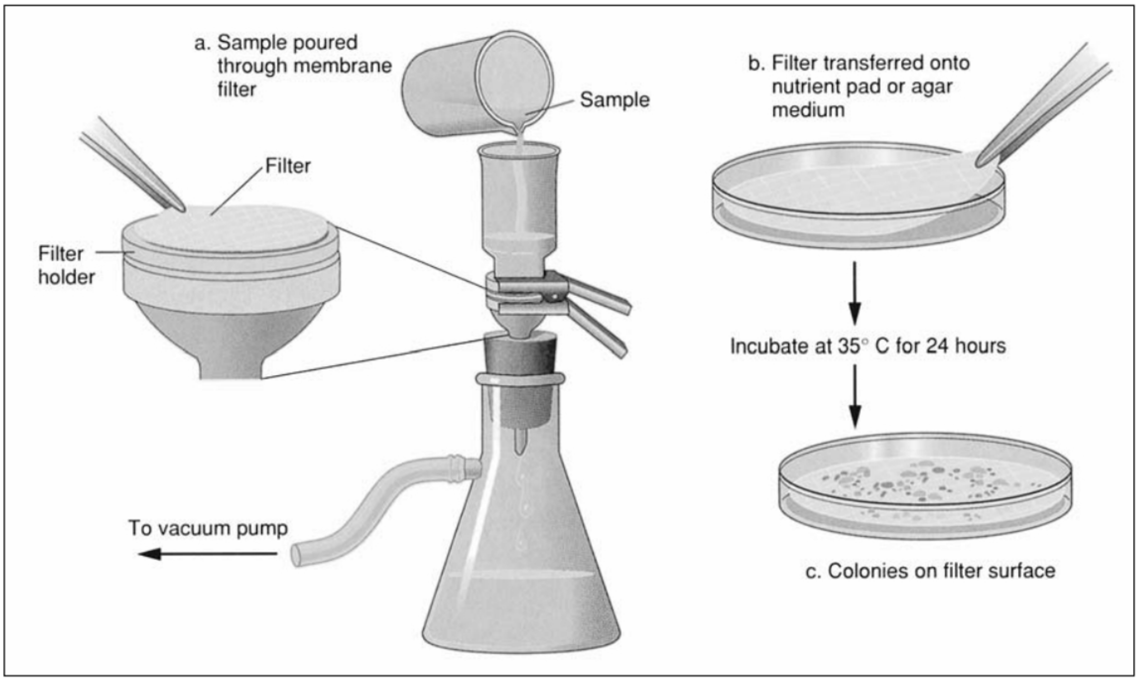
Water testing is used to analyze the quality of water. There are several methods that are used to make sure the water is safe for human use. Environmental Protection Agency (EPA) is the organization in charge of the regulations of the control of contamination of the drinking water, recreational, and aquatic water sources.

There are many harmless bacteria in water sources that do not cause any disease. The primary concern is the type of microorganisms present in the sources for drinking water. In particular, the presence of intestinal pathogens, such as *Vibrio cholera*, that can cause diarrheas and gastroenteritis is of concern.

The indicator used to detect fecal contamination of water in the United States is the bacterium *E. coli*. This marker organism suggests that possible pathogens might be present in the water, because *E. coli* is found in the human intestines and thus in human waste. *E. coli* is also a grouped as a coliform organism, which is a group of gram-negative, lactose fermenting, facultative anaerobe organisms.

The membrane filtration method is recognized by the US Public Health Services for the detection of *E. coli*/coliforms in water. It is a quantitative test and has the advantage of detection and enumeration. Greater sensitivity due to bigger volumes of water can be used, which allows for the detection of small numbers of bacteria.

22.1 Membrane Filtration.


a. Sample poured through membrane filter
Sample
Filter
Filter holder
To vacuum pump
b. Filter transferred onto nutrient pad or agar medium
Incubate at 35° C for 24 hours
c. Colonies on filter surface

Copyright © Kendall Hunt Publishing Company

Membranes with 0.22-μm pores are used to retain the microorganisms as the water passes through the pores. The membrane is placed onto the surface of a suitable culture media such as Eosin Methylene Blue and MacConkey + MUG agar plates. Colonies that grow can then be identified as coliforms based on the indicators in the media. MacConkey + MUG detects *E. coli* based on the presence of β-glucoronidase enzyme that fluoresce under UV light 355 nm.

MATERIALS

- ☑ Water samples from different sources
- ☑ Eosin methylene blue
- ☑ MacConkey + MUG agar (4 methylumbelliferyl-β-D-glucoronide)
- ☑ Membrane filtering unit
- ☑ 0.22-μm pore membrane
- ☑ Tubing
- ☑ Vacuum system
- ☑ Sterile graduate cylinders
- ☑ Sterile water in bottles
- ☑ Ethanol to clean filtering unit
- ☑ Forceps
- ☑ Aseptic burners

PROCEDURE

1. Assemble the membrane filtering unit as follows:
    a. Aseptically insert the filter holder base into the neck of a one liter side-arm flask.
    b. With a flamed forceps, place a sterile membrane filter disk, grid side up, on the filter holder base.
    c. Place the filter funnel on top of the membrane filter disk and secure it to the base with the clamp.
2. Attach the rubber hose to a vacuum source and pour the appropriate amount of water into the funnel (the amount of water depends on water quality. For waters with low turbidity, use 50 ml. For turbid waters, use 20 ml).
3. Disconnect the vacuum source, remove funnel, and carefully transfer the filter disks with sterile forceps to the Petri dish containing the desired culture media. Keep grid up.
4. Incubate at 37°C for 24 hours. Do not invert.

22.2 Colisure® (IDEXX) Test

This method is for detection and confirmation of total coliforms and *E. coli* in water. Any positive sample for total coliforms/*E. coli* is an indication of contamination of the water sample. The Colisure method can be applied to drinking water and ground water. It can be used as a Presence/Absence test, and it is a qualitative test.

The method is based on Defined Substrate Technology. It utilizes nutrient indicators that produce color/fluoresce when metabolized by total coliforms (Chromogenic substrate-β **galactosidase enzyme**) and *E.coli* (flourogenic substrate-β **glucoronidase enzyme**) It can detect bacteria at 1 CFU/100 ml at 24 hours. Positive results can be read in as little as 4 hours.

Coliform bacteria produce a red to magenta color and *E. coli* fluoresce under UV light at 355 nm.

MATERIALS

☑ Colisure vial
☑ Container for water sample
☑ 10 ml pipette
☑ 25 ml pipette
☑ Pipette helper
☑ UV light
☑ Face shield

PROCEDURE

1. The laboratory meeting prior to this exercise, obtain a specimen cup or other suitable container to bring a water sample of choice for this exercise.
2. Using a sterile pipette, add 10 ml of sample to vial with Colisure medium
3. Incubate for 24 hours at 37°C

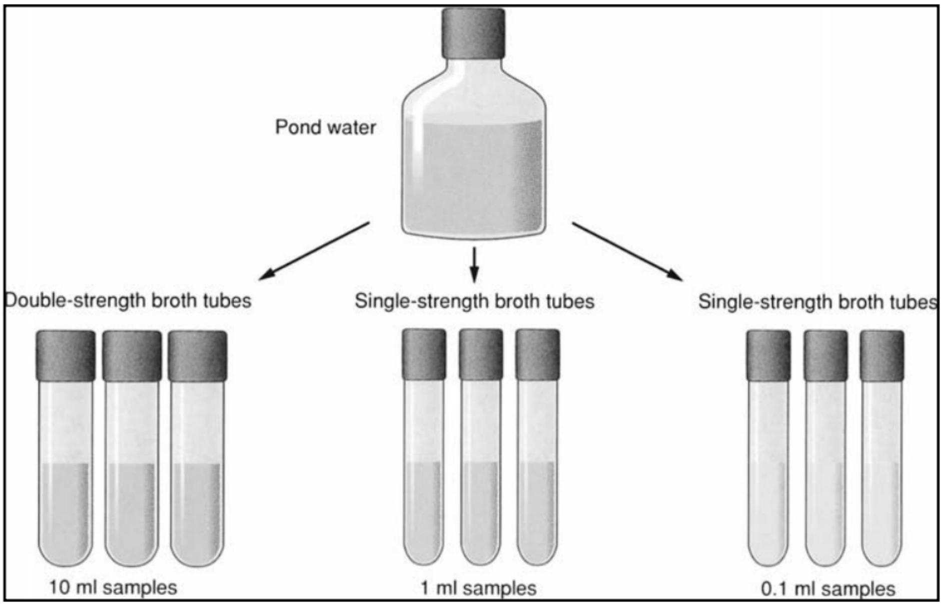
Appearance of Colisure vial	**Results**
Yellow/gold	Negative for total coliforms and *E. coli*
Red or magenta	Positive for total coliforms
Read/magenta and **fluoresce**	Positive for *E. coli*

22.3 Most Probable Number (MPN)

INTRODUCTION

The most probable number (MPN) method is a direct method of microbial growth. It is a statistical method to estimate the number of viable microorganisms in a sample. This method is based on the probability of a single cell that is present in a given amount of the sample. This is a great method to count bacteria that are present in small numbers.

In this exercise, Brilliant Green Bile Lactose Broth (BGBLB) tubes will be inoculated with different water or food samples. BGBLB is a selective medium for detection and enumeration of *E. coli* and coliform *(E. coli*-related organisms) based on the fermentation of lactose and gas production. *E. coli* are present in the feces of mammals including human beings. Their presence indicates fecal contamination and also means that pathogens could be present in a sample.


Pond water
Double-strength broth tubes
Single-strength broth tubes
Single-strength broth tubes
10 ml samples
1 ml samples
0.1 ml samples

Copyright © Kendall Hunt Publishing Company

MATERIALS

☑ Water samples from different sources brought by students
☑ 3 tubes with 10 ml of double strength (DS) BGBLB with Durham tube
☑ 3 tubes with 9 ml of BGBLB broth with Durham tube
☑ 3 tubes with 9.9 ml of BGBLB broth with Durham tube
☑ Sterile pipettes, 1 ml
☑ Sterile pipettes, 10 ml
☑ 90 ml sterile water in dilution bottles
☑ Eosin methylene blue agar plates
☑ Inoculating loop

PROCEDURE

1. Place 10 g of food sample in 90 ml of sterile water and shake well.
2. Aseptically transfer 10 ml of sample to each of the 3 tubes with 10 ml DS-BGBLB broth, label 10.
3. Aseptically transfer 1 ml of sample to each of the 3 tubes with 9 ml of BGBLB broth, label 1.
4. Aseptically transfer 0.1 ml of sample to each of the 3 tubes with 9.9 ml of BGBLB broth, label 0.1.
5. If the samples are milk, water of other liquid, proceed from step 2.
6. Incubate tubes at 37°C.
7. The presence of coliforms is indicated by a change of the indicator in the BGBLB tube or by the production of gas in the Durham tube. This is presumptive test for coliforms.
8. Using a Eosin methylene blue agar plate, divide the plate into 3 sectors and streak from the 3 tubes that have the highest dilution that were positive. Coliforms produce purple/red colonies upon growth with a red zone around them or metallic green sheen color. This is the confirmed test for coliforms. Further testing is necessary to identify the specific organisms.

LABORATORY REPORT 22
MEMBRANE FILTRATION AND COLISURE TEST

RESULTS

22.1 Membrane Filtration

Oserve the plates and identify the presence of any coliforms and/or *E. coli*

Eosine Methylene Blue

MacConkey + MUG (UV 355nm)

22.2 Colisure Test

Record and explain your results.

22.3 Most Probable Number

RESULTS

Sample tested ________________________________

Reactions for 10 ml ____ ______ _____

Reactions for 1 ml ____ ______ _____

Reactions for 0.1 ml ____ ______ _____

Determine Most Probable Number from Table Provided in Lab.

Results

______________coliforms per 100 ml of water.

QUESTIONS

1. Name one advantage and one disadvantage of the Colisure Test.

2. Name two advantages of the Membrane filtration test.

3. Name the sewage indicator organism used in the U.S.

4. Why is the Eeosine Methylene Blue used for the detection of coliforms?

5. List 2 characteristics of coliforms.

6. Name one advantage of the Compact Dry *E.coli* test

LABORATORY TWENTY-THREE

Microbial Interrelationships

LEARNING OBJECTIVES

Students will be able to:

- Describe and define the different types of symbiotic relationships
- Give an example of the different types of symbiotic relationship

PURPOSE

To observe different symbiotic relationships between different species of bacteria.

INTRODUCTION

Microorganisms are never found growing in a pure culture in nature. This is to be expected since all organisms are dependent on other organisms for survival. Such interrelationships may be beneficial for one or both of the participants in the association while other types benefit only one organism in the relationship. **Symbiosis** is the ability of organisms to live in a close association with a different type of organism. There are symbiotic situations in which symbionts have different types of relationships with each other, depending on the benefit derived by both members. Usually, the smaller of the two is the **symbiont** and the larger, the **host**.

There are several different types of symbiosis. In **mutualism** (sometimes called synergism or syntrophy), both organisms benefit from the association. Examples are provided to clarify the meaning of each association.

Example: *Rhizobium* bacteria which infect the roots of leguminous plants (endosymbionts). The *Rhizobium* bacteria are provided nutrients within the plant's roots and in turn, the bacteria fix nitrogen needed by the plants.

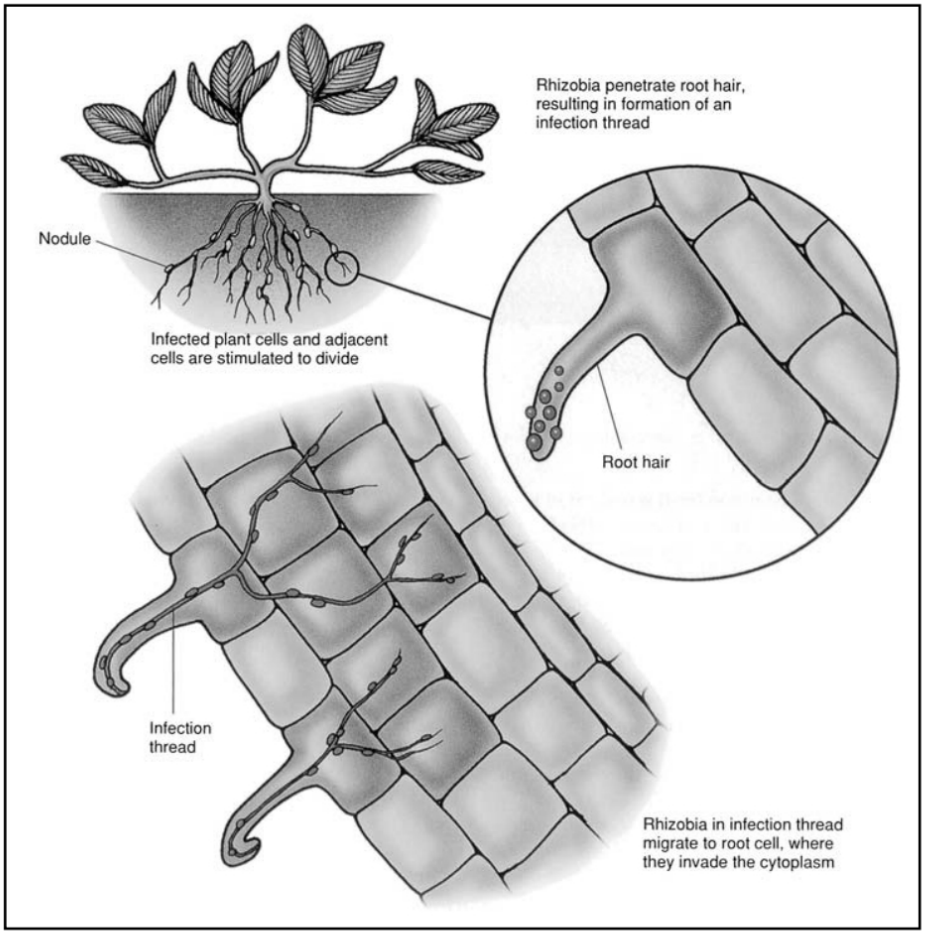
Commensalism is when one of the partners receives some benefit from the other, but the other is not harmed or helped.

Example: Strict anaerobes in the human colon benefit from depletion of the oxygen supply by facultative anaerobes while the facultative anaerobes receive nothing in return.

In a **parasitic** relationship, one symbiont benefits and the host is usually harmed.

Example: *Streptococcus pneumoniae* growth in human lung tissue can cause death of the host.

Antagonism involves the production of a substance, such as an antibiotic, by one member of the association which is detrimental to the second member.


Rhizobia penetrate root hair, resulting in formation of an infection thread
Nodule
Infected plant cells and adjacent cells are stimulated to divide
Root hair
Infection thread
Rhizobia in infection thread migrate to root cell, where they invade the cytoplasm

Copyright © Kendall Hunt Publishing Company

Example: *Bacillus* spp. produces an antibiotic that inhibits growth of the fungus, *Aspergillus*.

The effect of **competition** on two or more organisms in close proximity involves competition for nutrients and space. If one of the two organisms competing against each other does not occupy a unique position in the relationship, then competition occurs, usually to the detriment of one or both organisms.

The following table summarizes symbiosis (Atlas, 1997):

TABLE 23.1 Types of symbiosis.

Species Interaction	A	B
Mutualism	+	+
Commensalism	+	0
Parasitism	+	-
Antagonism (amensalism)	-	0 or -
Competition	-	-
Predation	+	-

0, no effect; +, positive effect; -, negative effect

There are four benefits that organisms may gain from an association with another type of organism:

1. **Protection.** Microorganisms in nature are exposed to hostile environments related to pH, osmolarity, temperature, water availability, presence or absence of oxygen and exposure to predators.

2. **Favorable position.** Some cyanobacteria and algae colonize fungi to form a second entity called a lichen. The fungi hold onto the photosynthetic cyanobacterium or alga so that they are exposed to sunlight and not swept away by wind or water.

3. **Nutrition.** One or both members of the association provide growth factors required for growth. One or both symbiont members may benefit as in infection of leguminous plants by nitrogen-fixing bacteria.

4. **Recognition devices.** Bioluminescent microorganisms which infect organs of deep-sea fish serve as recognition devices for the fish in schooling, mating and attracting prey.

A group of bacteria that form a somewhat loosely-knit association of organisms in the environment is called a **consortium**.

In these exercises, we will observe several different types of microbial interrelationships.

LABORATORY EXERCISE

23.1 Symbiosis: Rhizobium.

MATERIALS

- ☑ Leguminous plant with root system(such as bluebonnet or clover)
- ☑ methylene blue
- ☑ razor blade
- ☑ microscope slide
- ☑ coverslip
- ☑ inoculating loop
- ☑ immersion oil
- ☑ bibulous paper
- ☑ lens paper

PROCEDURE

1. Observe the root system of the leguminous plant. Note the root nodules (if more than one type of plant is available, compare differences in the nodules).
2. Slice open a nodule with a razor blade. Observe any color in the exposed nodule.
3. Make a smear on a microscope slide by pressing the open side of the nodule on the center of the slide. Prepare the smear for a Simple Stain (see Exercises 6.1 and 7.1) using methylene blue. Observe on oil immersion.
4. Slice a thin section of the nodule and place in the center of a slide. Add a drop of water and coverslip to prepare a wet mount (see Exercise 1.1). Observe at 100X and 400X magnification.

23.2 Synergism.

ORGANISMS

(liquid culture in tube)

☑ *Escherichia coli*

☑ *Proteus vulgaris*

☑ *Staphylococcus saprophyticus*

SUPPLIES

☑ 3 lactose broth tubes with Durham tubes and bromothymol blue indicator

☑ 3 sucrose broth tubes with Durham tubes and bromothymol blue indicator

☑ inoculating loop

PROCEDURE

Each lab group will inoculate using one of the following sets of organisms as assigned by the teaching assistant: *E. coli* and *P. vulgaris; E. coli* and *S. saprophyticus*.

1. Label one lactose broth and one sucrose broth for each of the two organisms assigned. Label a third tube of each type broth “mixed”.
2. Inoculate each tube with one loopful of the appropriate organism. To the mixed tube, inoculate with one loop of both of the assigned organisms.
3. Incubate the six tubes at 37°C for 48 hours.
4. Observe growth, color, and gas production in each tube. Note if the mixed culture produced results that neither culture could alone. Obtain results for the other organism pairs from other lab groups.

23.3 Commensalism.

ORGANISMS

(liquid culture in tube unless otherwise noted)

☑ *Escherichia coli*

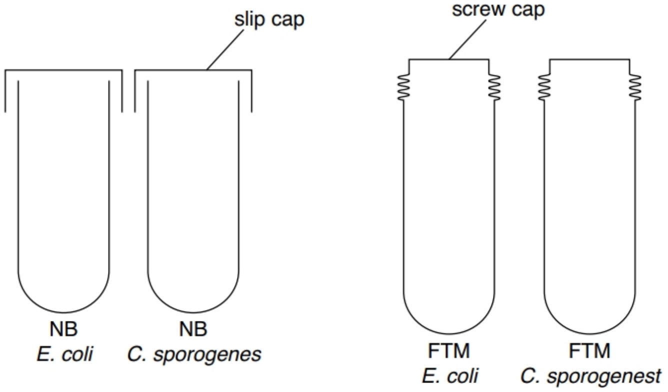
☑ *Clostridium sporogenes* in fluid thioglycollate medium

MATERIALS

- ☑ 3 nutrient broth tubes
- ☑ 2 fluid thioglycollate medium tubes
- ☑ inoculating loop
- ☑ Transfer pipette

PROCEDURE

1. Label one nutrient broth tube and one fluid thioglycollate medium tube each with the name of the two organisms. Label a third nutrient broth tube with “mix of *Escherichia coli* and *C. sporogenes*”.
2. Inoculate the appropriate tubes with one loopful of *E.coli*.
3. Transfer 0.1 ml of *C. sporogenes* into the appropriate nutrient broth tubes. Inoculate the fluid thioglycollate medium tube with a loopful of *C. sporogenes*.
4. Incubate all tubes at 37°C for 48 hours.
5. Observe growth in all tubes. Note pattern of growth in the fluid thioglycollate tubes without disturbing. Swirl the nutrient broth tubes to disperse the cells. Take a sample from all tubes and prepare a smear for Gram staining (see Exercises 6.1 and 8.1). Note the types of cells present in each sample.



add 3 drops of *E. coli*
3 drops of *C. sporogenes*

LABORATORY REPORT 23
MICROBIAL INTERRELATIONSHIPS

Name:______________________________________

RESULTS

23.1 Symbiosis: Rhizobium.

Describe or sketch below:

Rhizobium Gram stain	*Root Nodule* Microscopic	*Root Nodule* Macroscopic

23.2 Synergism.

Organisms used:

1) _________________________ and 2) ____________________________

Describe growth, color of media, and any gas production in:

Lactose broth Organism #1:

____

Lactose broth Organism #2:

____

Lactose broth Mixed:

Sucrose broth Organism #1:

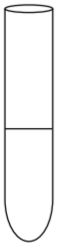
Sucrose broth Organism #2:

Sucrose broth Mixed:

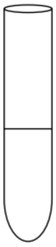
23.3 Commensalism.

	Describe growth in Nutrient Broth	Gram stain
Escherichia coli		
C.sporogens		
E.coli AND C.sporogenes		

Draw growth in fluid thioglycollate medium:



E.coli



C. sporogenes

QUESTIONS

1. Define parasitism: ____

   ____

2. In ____________________________, one organism benefits and the other is not affected.

3. Give an example of a mutualistic symbiosis and explain what each organism gains from the relationship:

   ____

   ____

4. Which type of relationship is most likely to occur between two different organisms in the environment which are competing for the same resources?

SECTION SEVEN

Microbial Genetics

LABORATORY TWENTY-FOUR

Transformation

LEARNING OBJECTIVE

Students will be able to:

- Describe the process of transformation and interpret the results of a transformation experiment.

PURPOSE

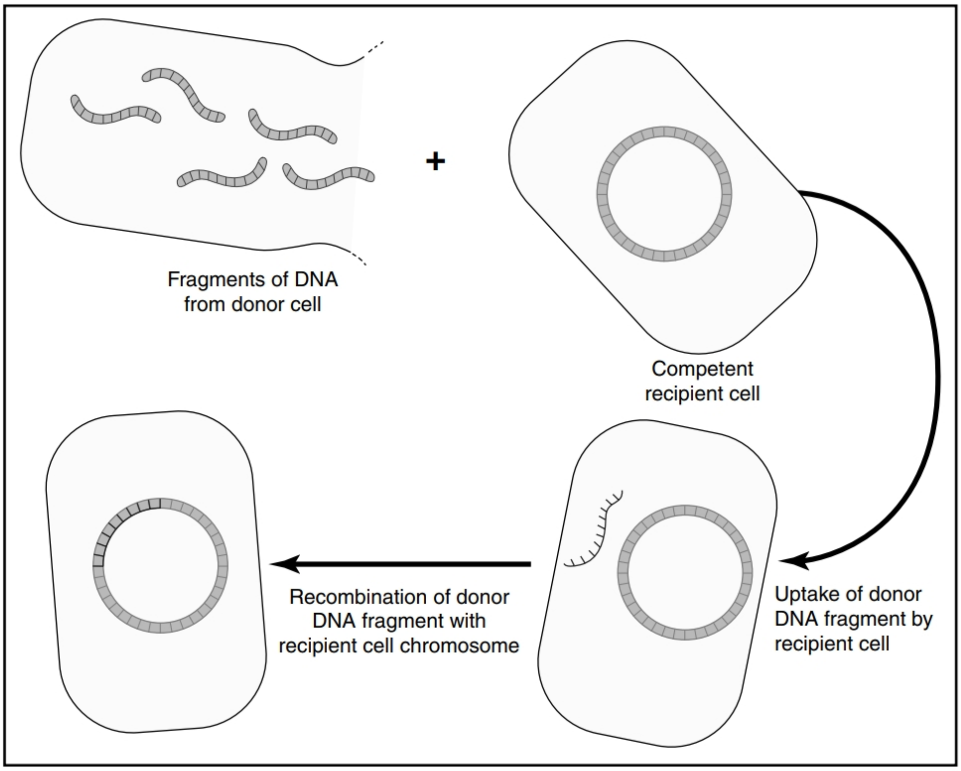
To observe natural transformation in bacteria.

INTRODUCTION

In 1928, Fred Griffith observed that non-virulent *Streptococcus* became virulent when injected into mice along with a heat-killed virulent strain of *Streptococcus*. Only virulent bacteria were recovered from the dead mice. **Transformation** is the term Griffith gave this change of non-virulent bacteria into virulent strains. These experiments by Griffith were pioneering work which set the stage for later experiments that proved DNA was the genetic material. It is now known that transformation is the uptake of naked DNA by the cell. Cells which are able to take in naked DNA are said to be **competent**.

Several genera besides *Streptococcus* are naturally competent. One of these is *Acinetobacter*, a common soil organism. Other bacteria, such as *E. coli*, can be made artificially competent through chemical treatment. One procedure is the treatment of the cells with magnesium chloride which makes their membranes more permeable to DNA. Techniques such as these are used frequently in molecular biology for introduction of genes into bacteria.

In this experiment, you will observe natural transformation in *Acinetobacter* spp. Two strains, *Acinetobacter* ADP1 and *Acinetobacter* ADP6, will be used. The *Acinetobacter* ADP1 strain is a wild-type strain which is capable of degrading p-hydroxybenzoate (POB) as a carbon source. Breakdown of a compound for use as carbon and/or energy is a catabolic process. The *Acinetobacter* ADP6 strain is a mutant which lacks the enzyme protocatechuate oxygenase, a required enzyme in the pathway for utilization of POB. Strain ADP6 will grow well on minimal medium containing glucose as a carbon source, but cannot grow when POB is the sole carbon source on the plate. Transformation will be demonstrated by lysing (breaking) the cells from *Acinetobacter* ADP1 by using TENS solution that contains Tris buffer, ethylenediamine tetraacetic acid (EDTA), sodium hydroxide (NaOH) and sodium dodecyl sufate (SDS). The Tris-buffer provides a pH buffer to help maintain the DNA, while EDTA is a chelating agent which removes divalent cations (*i.e.* Mg^{++}) that are required by DNA degrading enzymes.


Fragments of DNA from donor cell
+
Competent recipient cell
Uptake of donor DNA fragment by recipient cell
Recombination of donor DNA fragment with recipient cell chromosome

Copyright © Kendall Hunt Publishing Company

The sodium hydroxide breaks the cell wall, and the SDS is a detergent which solubilizes the cell membrane. and placing the resulting lysate with *Acinetobacter* ADP6 cells onto a minimal plate containing POB as the carbon source. Only those ADP6 cells which take in DNA containing a functional gene for protocatechuate oxygenase will grow.

LABORATORY EXERCISE

24.1 Transformation.

ORGANISMS

(liquid culture in tube unless otherwise noted)

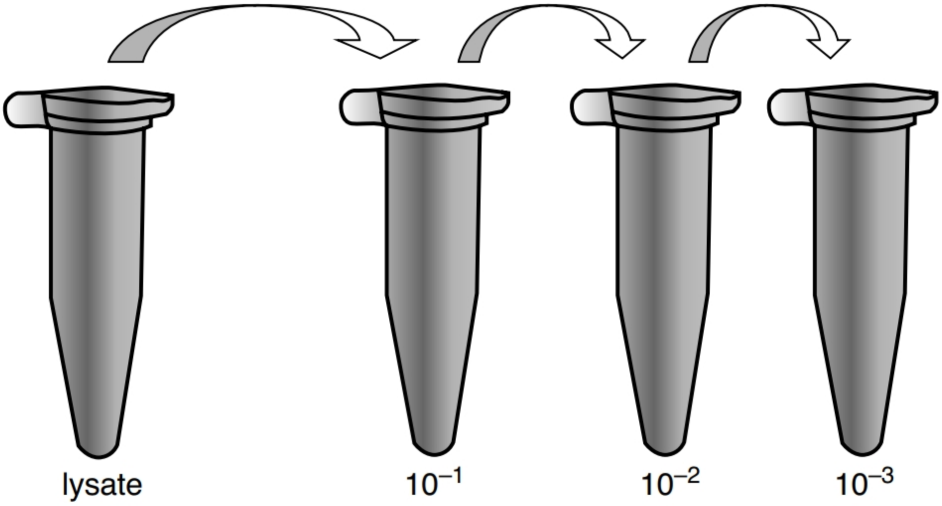
☑ *Acinetobacter* ADP1 (lysate)
☑ *Acinetobacter* ADP6

MATERIALS

☑ 1 Nutrient agar
☑ 3 *p*-hydroxybenzoate (POB) plates
☑ microcentrifuge
☑ microcentrifuge tubes
☑ micropipetters
☑ turntable
☑ cell spreader
☑ ethanol
☑ TENS solution

PROCEDURE

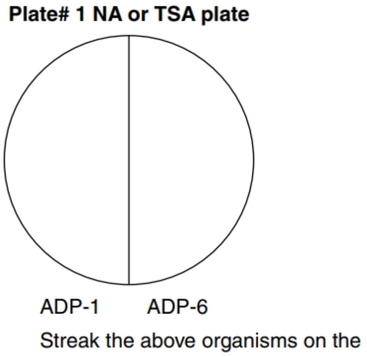
1. Prepare ADP–1 lysate (alkaline lysis)
    - Collect cells from 1.5 ml of culture by centrifugation for 2 min 10K rpm
    - Discard supernatant in biohazard bag
    - Add 300 μl of TENS solution, mix by vortex for 2–5 seconds
2. Prepare dilutions of the ADP 1 lysate
    - Use empty sterilized microcentrifuge tubes and make 10^{-1}, 10^{-2} and 10^{-3} dilution of the lysate by adding 100 μl of the filtrate to 900 μl sterile water or saline solution.



a. Aseptically add 900 μL of **sterile water** to each of the three tubes (10^{-1}, 10^{-2}, and 10^{-3})
b. Transfer 100 μL of your lysate (undiluted) to the tube labeled 10^{-1}. Mix by vortexing, discard the pipette tip
c. Transfer 100 μL from your 10^{-1} tube to your 10^{-2} tube. Mix by vortexing, discard the pipette tip
d. Transfer 100 μL from your 10^{-2} tube to your 10^{-3} tube. Mix by vortexing, discard the pipette tip

3. Plate the following:
    - A. **Nutrient Agar plate control plate (#1)**. Divide plate into two quadrants, inoculate with a single streak *Acinetobacter* ADP1 in one side of the plate, and *Acinetobacter* ADP6 on the other half.
    - B. **POB plate (#2)**. control plate. Divide plate into two quandrants, inoculate with a single streak *Acinetobacter* ADP1 in on side of the plate , and *Acinetobacter* ADP6 on the other half.
    - C. **POB plate (#3)**. divide plate into four quadrants and label 10°, 10^{-1}, 10^{-2} and 10^{-3}. Drop 10 μl of *Acinetobacter* ADP1 lysate at each corresponding dilution into its respective quandrant. This is also a control. This will show if the ADP1 cells were completely lysed (any colonies mean incomplete lysis) KEEP PLATES FLAT UNTIL DRY.
    - D. **POB plate (#4)**. again divide plate and label as in B. above. Spread (see Exercise 11.2) with 100 μl *Acinetobacter* ADP6 cells. Wait 5 minutes for plate to dry. Then, as in C, drop 10 μl of each dilution of *Acinetobacter* ADP1 lysate into the respective quadrants. This is the experimental plate. Any colonies that grow here are a result of ADP6 cells taking up ADP1 DNA, specifically the piece of DNA (gene) that codes for the enzyme protocatechuate oxygenase. The number of colonies would be expected to decrease with a decrease in the amount of DNA (diluted DNA) dropped onto the plate. KEEP PLATES FLAT UNTIL DRY.
4. Incubate all plates at 37°C for 24–48 hours.
5. Note growth and number of colonies on plates.

FIGURE 24.1 Transformation.


Plate# 1 NA or TSA plate
ADP-1
ADP-6

Streak the above organisms on the

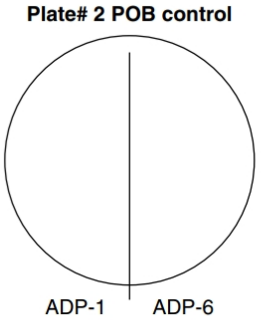

Plate# 2 POB control
ADP-1
ADP-6

FIGURE 24.2 Transformation plate 3.

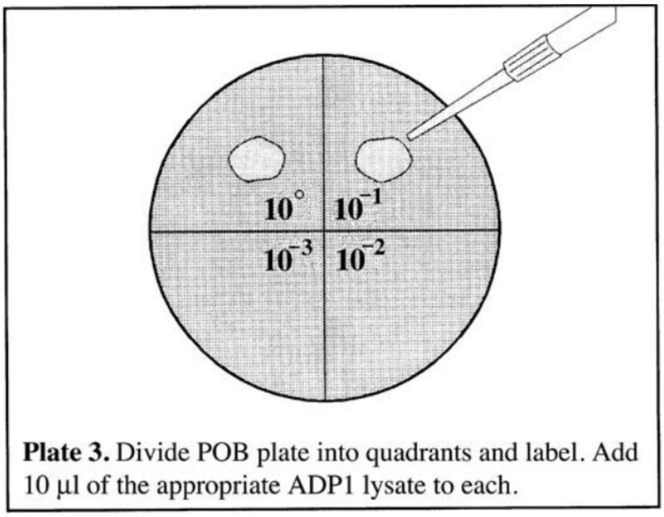

10°
10^{-1}
10^{-3}
10^{-2}

Plate 3. Divide POB plate into quadrants and label. Add 10 µl of the appropriate ADP1 lysate to each.

FIGURE 24.3 Transformation plate 4.

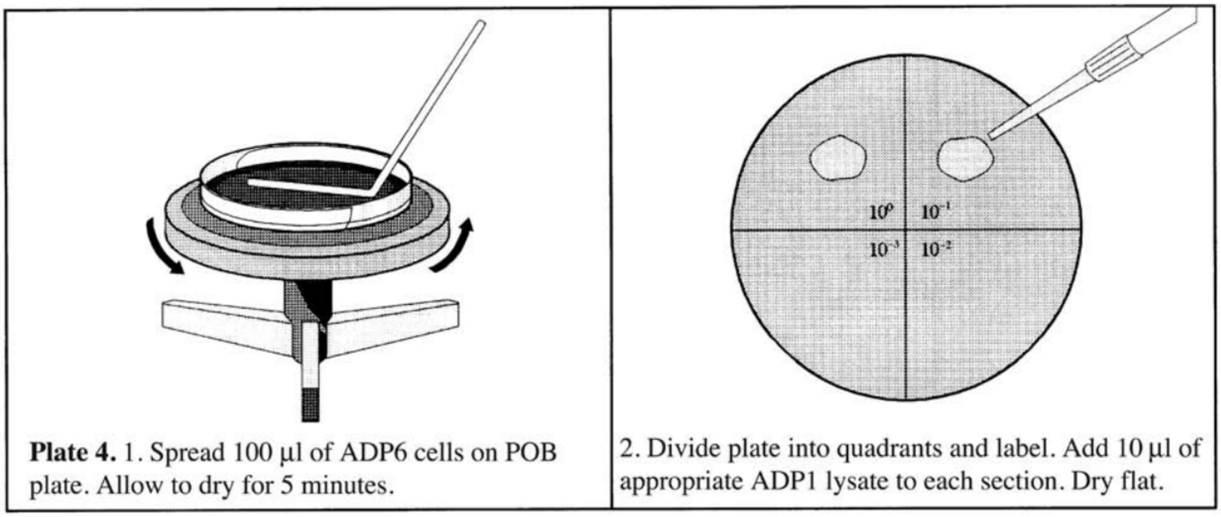

10^0
10^{-1}
10^{-3}
10^{-2}

Plate 4. 1. Spread 100 µl of ADP6 cells on POB plate. Allow to dry for 5 minutes.

2. Divide plate into quadrants and label. Add 10 µl of appropriate ADP1 lysate to each section. Dry flat.

ARTIFICIAL TRANSFORMATION OF ESCHERICHIA COLI

LEARNING OBJECTIVE

Students will be able to make a bacterial culture artificially competent and successfully transform the cells with a plasmid.

PURPOSE

Make *Escherichia coli* competent by $CaCl_2$ treatment to see transformation

ORGANISMS

Escherichia coli

pUC 19 plasmid

MATERIALS

- ☑ $CaCl_2$ ice cold
- ☑ TSA and TSA + Ampicillin (50µg/ml)
- ☑ Microcentrifuge
- ☑ Microcentrifuge tubes
- ☑ Micropipetters
- ☑ Pipette tips
- ☑ Water bath

PROCEDURE

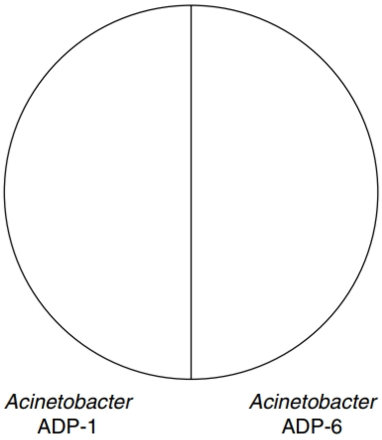
1. Add 1.5ml of *Escherichia coli* cultures to a 1.5ml microcentrifuge tube.
2. Centrifuge at 8,000 rpm for 5 minutes
3. Discard supernatant
4. Repeat Step 1-3 twice
5. Re-suspend pellet in 1ml ice cold $CaCl_2$
6. Add 2ul of pUC 19 plasmid
7. Place on ice (bucket) for 15 min
8. Place microcentrifuge tube on water bath at 42ºC for 1.5 minutes
9. Remove from water bath and place on ice (bucket) for 2 minutes
10. Add 500ul of tryptic soy broth (TSB) to the tube
11. Place in shaker incubator at 37ºC for 45 minutes
12. Spread plate 100ul of sample on selective plate (TSA + Ampicillin 50mg/ml)
13. Incubate plates overnight at 37ºC

LABORATORY REPORT 24
TRANSFORMATION

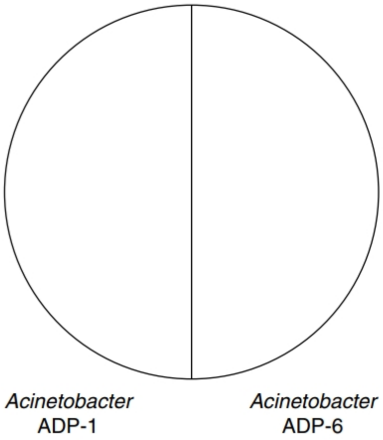
Name:______________________________________

RESULTS

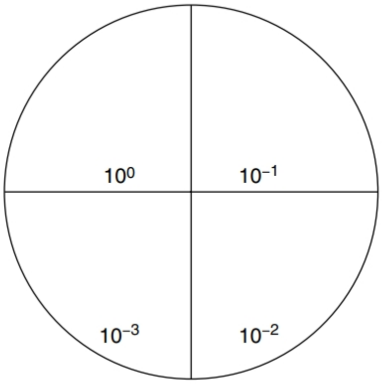
A. Draw and describe growth on Nutrient agar Plate


Acinetobacter
ADP-1
Acinetobacter
ADP-6

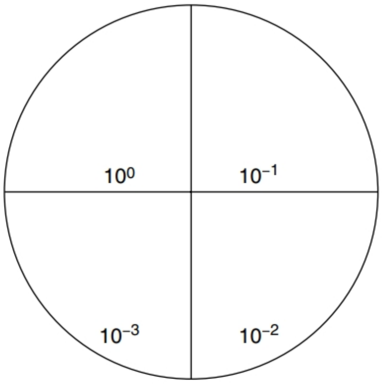
B. Draw and describe growth on POB plate


Acinetobacter
ADP-1
Acinetobacter
ADP-6

C. Draw and describe growth on POB Plate #1—*Acinetobacter* ADP1 lysate only:


10^0
10^{-1}
10^{-3}
10^{-2}

D. Draw and describe growth on POB Plate #2—*Acinetobacter* ADP6 cells plus *Acinetobacter* ADP1 lysate:


10^0
10^{-1}
10^{-3}
10^{-2}

LABORATORY REPORT

ARTIFICIAL TRANSFORMATION OF *ESCHERICHIA COLI*

Name:______________________________

RESULTS

Observe growth of *Escherichia coli* on triptic soy agar plates with **ampicillin 50mg/ml**

A. *E.coli* cultures treated with $CaCl_2$ to make *E.coli* competent

B. Untreated *E.coli* cultures.

QUESTIONS

1. Define transformation: ____

____

2. *E. coli* can be made artificially competent through ______________________.

3. The utilization of p-hydroxybenzoate is a(n) ________________________ process.

4. Name a genus which is naturally competent: ___________________

5. Which plate in the experiment was a negative control to insure that ADP1 cells were completely lysed? ____________________

6. Which strains of *Acinetobacter* where used for the transformation experiment?

LABORATORY TWENTY-FIVE

Genetic Mating: Conjugation

LEARNING OBJECTIVES

Students will be able to:

- Describe the process of conjugation
- Interpret the results of a conjugation experiment.

PURPOSE

To observe the transfer of genetic material between bacteria through conjugation.

INTRODUCTION

Bacteria are not capable of sexual reproduction, but they do possess a form of genetic transfer which is the equivalent of bacterial sex. **Conjugation** is a unidirectional transfer of genetic material which requires cell to cell contact. This transfer requires the presence of a conjugable plasmid. A **plasmid** is closed-circular, extrachromosomal DNA capable of self-replication. A conjugable plasmid contains a set of genes designated the *tra* genes which are necessary to transfer the plasmid from the donor to the recipient. The transfer of the plasmid occurs while the donor and recipient cells are held close together by a structure called a **pilus**. The pilus proteins are encoded on the plasmid and form a rod-like structure on the surface of the donor cell. The pilus holds the recipient cell to the donor cell while DNA transfer occurs. The transfer of the plasmid DNA is replicative and unidirectional. As the plasmid is copied, the copy is transferred to the recipient cell. When conjugation is complete, both the donor and the recipient contain copies of the plasmid.

In this exercise, conjugation will be observed between two strains of *Pseudomonas putida*. The donor strain, *P. putida* PAW15, is a leucine auxotroph (requires the amino acid leucine for growth) which contains the TOL plasmid. The TOL plasmid is a conjugable plasmid which also contains genes for the catabolism of *m*-toluate. The phenotype of this strain, Leu^-Tol^+, indicates that *P. putida* PAW15 will grow on a minimal medium containing *m*-toluate as the sole carbon source, but only if leucine is also added. The recipient strain, *P. putida* 503CA, is a prototroph (is able to synthesize the amino acids it needs through **anabolic** processes), but does not contain the TOL plasmid. In comparison to strain PAW15, *P. putida* 503CA has a phenotype of Leu^+Tol^- and will grow on glucose minimal medium without any additions. It cannot, however, grow on a minimal medium plate with *m*-toluate as the sole carbon source. Following the procedures outlined below, these two strains will be tested to confirm their phenotypes and then crossed on an experimental plate to see the results of conjugation. This experimental plate is chosen to provide an environment on which neither of the parent cells (donor or recipient) can survive, but on which the recombinant daughter cells (recipient plus plasmid) will grow.

LABORATORY EXERCISE

25.1 Conjugation.

ORGANISMS

(slant or plate culture)

☑ *Pseudomonas putida* PAW15

☑ *Pseudomonas putida* 503CA

☑ *Escherichia coli*

MATERIALS

☑ 1 Glucose minimal plate

☑ 1 *m*-toluate minimal + leucine plate

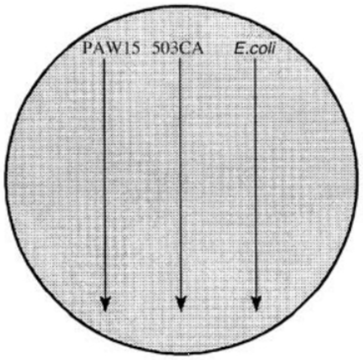
☑ 1 *m*-toluate minimal plate

☑ inoculating loop

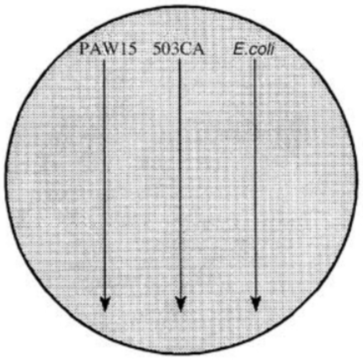
PROCEDURE

1. Make three parallel streaks, one of each organism, on the glucose minimal plate. This is a control plate.
2. In the same manner as the first plate, make three parallel streaks on the *m*-toluate + leucine plate. This is another control plate.
3. The experimental plate is the *m*-toluate plate. First, make a single streak of *P. putida* PAW15 along the left side of the plate. Next, make a single streak of *P. putida* 503CA from left to right which crosses the first streak. Repeat with a parallel, single streak of *E. coli* which also crosses the first streak.
4. Incubate the plates at 30°C for 4–7 days.
5. Observe the growth on each plate. Based on known phenotypes, determine if the actual results for the controls match the expected. Sketch and explain the results for each plate.

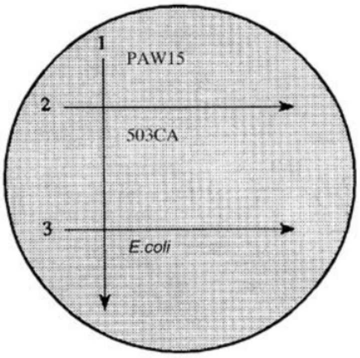
FIGURE 25.1 Conjugation plates.


PAW15
503CA
E.coli

1. Glucose minimal plate: Make three parallel streaks, one of each organism. Write organism's names.


PAW15
503CA
E.coli

2. *m*-toluate minimal + leucine plate: Make three parallel streaks, one of each organism. Write organism's names.


1
PAW15
2
503CA
3
E.coli

3. *m*-toluate minimal plate: Make three streaks in order as shown. 1 = PAW15; 2 = 503CA; 3 = *E. coli*.
Write organism's names.

LABORATORY REPORT 25
CONJUGATION

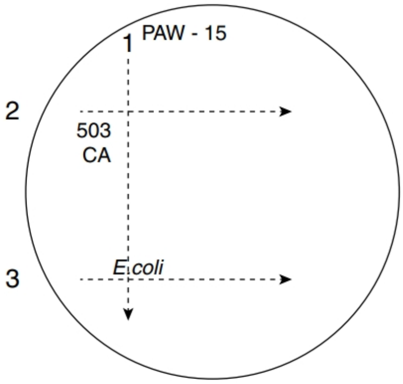
Name:______________________________________

RESULTS

A. Draw and describe the growth on the Glucose minimal plate:

B. Draw and describe the growth on the *m*–Toluate + leucine plate:

C. Draw and describe the growth on the *m*–Toluate plate:



QUESTIONS

1. The synthesis of required compounds from scratch (i.e. basic carbon and nitrogen sources) is a(n) ____________________ process.

2. Conjugation requires ____.

3. True or False. Neither of the parent cells can grow on the experimental plate.

4. *P. putida* PAW15 requires _______________________ for growth.

5. The genes encoding the proteins necessary for transfer are found on a __________________.

6. What is the phenotype of the daughter cells? ________________

7. **Fill in description**

Microorganism/plasmid	**Phenotype**	**Characteristic**
Pseudomonas putida PAW 15		
Pseudomonas putida 503CA		
Escherichia coli		
TOL plasmid		

LABORATORY TWENTY-SIX

Bacteriophage

LEARNING OBJECTIVE

Students will be able to:

- Identify plaques produced by bacteriophage infection on a bacterial lawn.

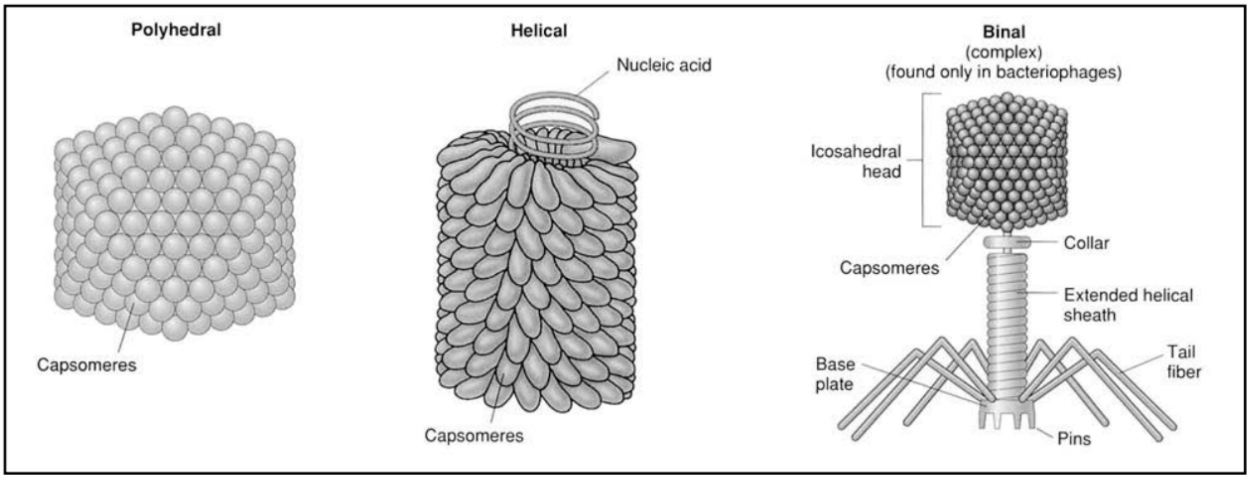
PURPOSE

To observe the the infection of bacteria by bacteriophages.

INTRODUCTION

Viruses are noncellular, obligate intracellular parasites. Viruses exist in many shapes and sizes. They contain a either DNA or RNA as their genetic material. The genetic material is often enclosed in a protein coat called the **capsid**, and may also contain a tail or body through which the genetic material is injected into the host. At the end of this structure, tail fibers are present which have the ability to attach to specific sites, called **receptors**, on the host cell (Fig. 26.1). Receptors are generally proteins or glycoproteins on the surface of the cell. Viruses which infect bacteria are called **bacteriophage**, or simply phage (*phagein*, to eat). Phage which specifically infect *E. coli* are collectively referred to as coliphages.

FIGURE 26.1 T4 bacteriophage.


Polyhedral
Capsomeres
Helical
Nucleic acid
Capsomeres
Binal
(complex)
(found only in bacteriophages)
Icosahedral head
Capsomeres
Collar
Extended helical sheath
Base plate
Tail fiber
Pins

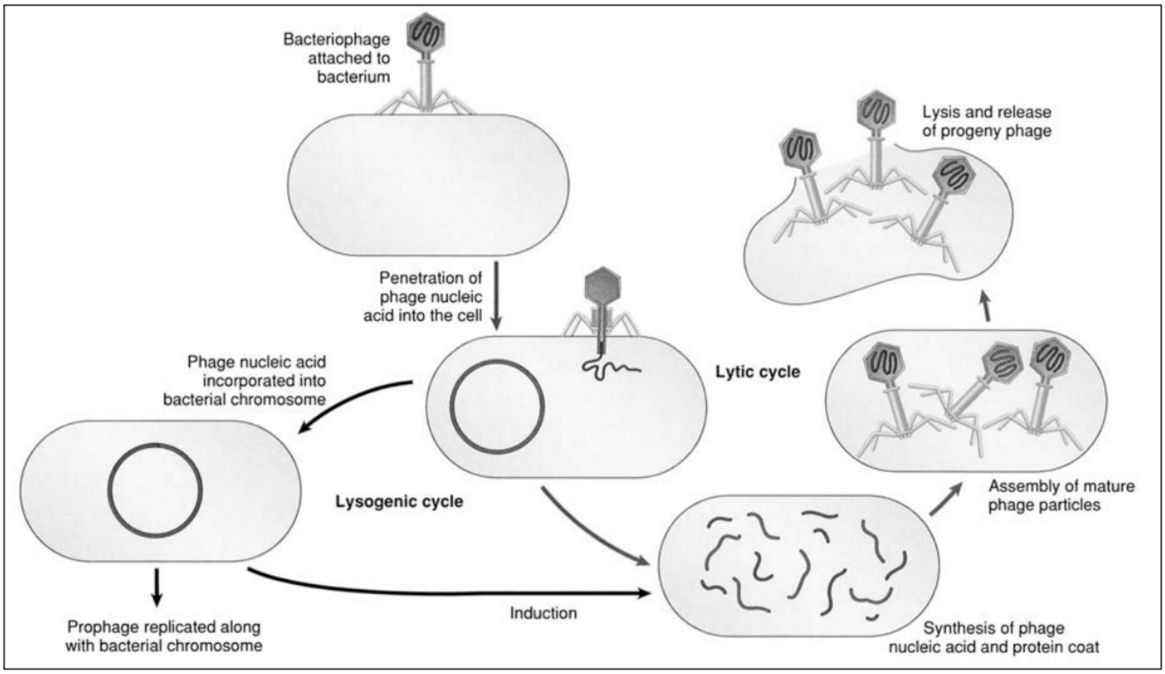
Copyright © Kendall Hunt Publishing Company

Infection by phage can follow two courses, lysis or lysogeny. A **lytic** phage causes lysis of the host cell by first taking over the replicative machinery of the cell and then using these resources for creation of the components for new phage. These components are assembled into mature phage. Once the cell resources have been depleted, the host cell bursts, and the phage particles are released to infect other bacterial cells. In **lysogeny**, the majority of infections follow the lytic cycle described above. However, a small percentage of phage infections form a stable relationship with the host cell. In these, the phage incorporates into the genome of the host at a specific site. This relationship is called a lysogen. Lysogenic bacteria grow and replicate normally for any number of generations. Yet, during times of cellular stress, the phage can remove itself from the genome and enter the lytic cycle.

Bacteriophage infections can be visualized by the formation of **plaques**, zones of clearing, in bacterial lawns. Lytic phages produce clear plaques, due to killing of all the cells in the zone, while lysogenic phages give cloudy plaques because of the growth of lysogens within the zone. Since each plaque is produced by a single viral particle or plaque-forming unit (PFU), plaques can be used to count the number of bacteriophage present. The number of bacteriophage in a sample is known as the **titer**.

In this exercise, you will use a provided sample containing phage. The sample will be used to infect a host strain, and the plaques formed by this infection will be observed on a pour plate preparation.

FIGURE 26.2 Lytic and Lysogenic cycle of viruses


Bacteriophage attached to bacterium
Lysis and release of progeny phage
Penetration of phage nucleic acid into the cell
Phage nucleic acid incorporated into bacterial chromosome
Lytic cycle
Lysogenic cycle
Assembly of mature phage particles
Prophage replicated along with bacterial chromosome
Induction
Synthesis of phage nucleic acid and protein coat

Copyright © Kendall Hunt Publishing Company

LABORATORY EXERCISE

26.1 *Growing viruses*

ORGANISMS

(liquid culture in tube)

☑ *Escherichia coli* B

MATERIALS

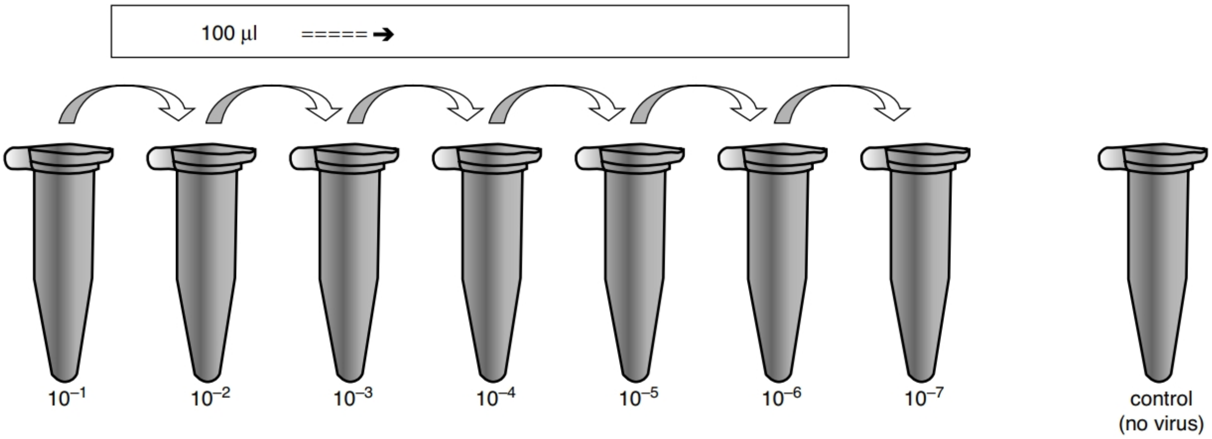
☑ centrifuge and sterile tubes
☑ sterile tubes, slip top
☑ pipette helper
☑ tubes tryptone soft agar, 4 ml
☑ tryptone agar plates
☑ 1000 µl micropipette
☑ 100 µl micropipette
☑ pipette tips
☑ sterile water for dilution

PROCEDURE

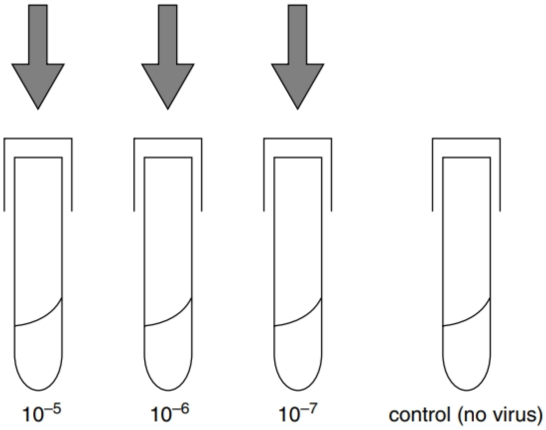
(Wear gloves at all times)

1. Each table will be provided with T4 bacteriophage at assigned dilution.

2. Dilute phage by using sterile microcentrifuge tubes to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} dilution of the phage by adding 100 µl to 900 µl of **phage buffer**.



3. Obtain 5 sterilized glass tubes and label with different dilutions and control. Add 100µl of each dilution to the tubes except to the control tube.



4. Add 0.1ml of *E. coli* B to all tubes
5. Incubate all tubes at 37° C in a incubator for 10 minutes
6. Add 0.1 ml of *E. coli* B (lawn cells) to each tube.
7. One at the time, add 4ml of tryptone soft agar (60°C) to the tube of virus-bacteria mix and then immediately pour onto **tryptone agar plate (plates labeled in advance with correct dilutions),** swirl on a flat surface to spread evenly, and allow to solidify. Repeat for each tube.
8. Incubate at 37° C.

LABORATORY REPORT 26
BACTERIOPHAGE

Name:_______________________________________

RESULTS

1. Describe the growth and plaque formation on the solid media used.

2. Calculate __________________________________ the Titer

QUESTIONS

1. A ______________________ is a zone of clearing in a bacterial lawn caused by a bacteriophage.

2. ______________________ is the general name given to bacteriophage which specifically infect *E. coli*.

3. In the _____________________ cycle, the phage can incorporate into the genome of the host cell.

4. The genetic material is found within the __________________ of the phage.

5. Why were additional *E. coli* cells added to the phage mixture after the first incubation?
   ____

   ____

SECTION EIGHT

Biochemical Tests for Identification

The purpose of completing the different biochemical tests using pure cultures of microorganisms is to determine the identity of the microorganism being tested. Many bacteria produce enzymes, acids and gases that are unique to the genus. Some tests are species-specific. Medical laboratory scientists use the results of the distinctive biochemical reactions to aid physicians in prescribing relevant drug therapies for people infected by pathogenic bacteria. Following are some of the biochemical tests for differentiation of pathogens and normal bacterial microbiota.

LABORATORY TWENTY SEVEN

IMVC

LEARNING OBJECTIVES

Students will be able to:

- Describe results of the IMVC test
- Interpret the IMVC test to identify bacteria

PURPOSE

To understand the use of IMVC test in bacterial identification

INTRODUCTION

The **IMVC** tests are a series of physiological tests, usually for coliform bacteria detection, to differentiate *E. coli* bacteria (indicative of fecal contamination) from *Enterobacter* (no fecal contamination).

The results of the IMVC tests for the *E. coli* and *Enterobacter* bacteria are:

	I	**M**	**V**	**C**
E. coli	+	+	-	-
Enterobacter	-	-	+	+

For the laboratory exercise, each student will select one or more of the following organisms and perform all four of the IMVC tests.

ORGANISMS

(liquid culture in tube)

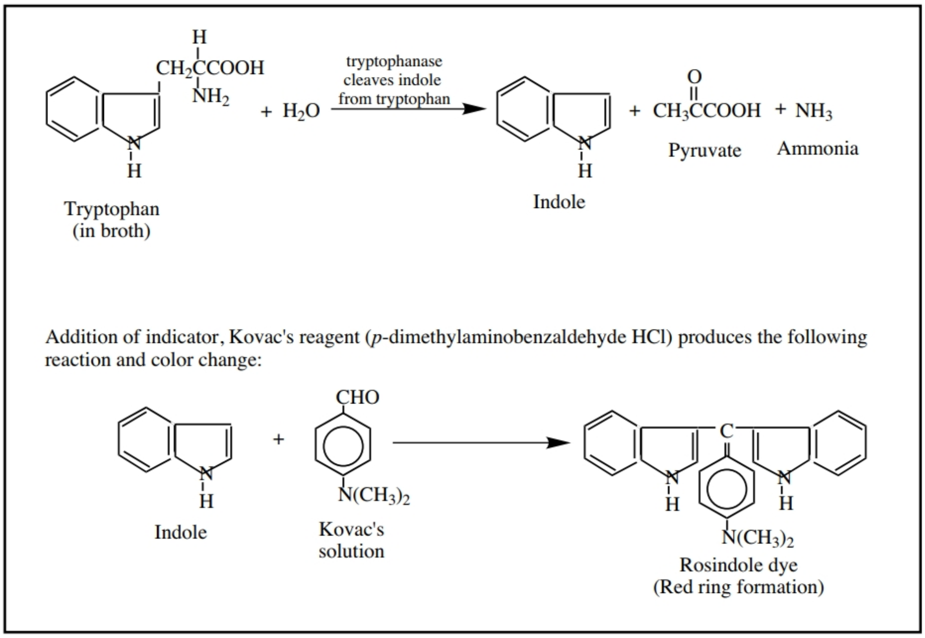
☑ *Citrobacter freundii*
☑ *Escherichia coli*
☑ *Pseudomonas fluorescens*
☑ *Morganella morganii*
☑ *Serratia marcesceus*
☑ *Providencia stuartii*
☑ Other Enterobacteria

27.1 Indole Oxidation.

INTRODUCTION

The Indole test is the first of the four tests in the IMVC series. It is used to differentiate between enteric bacteria which are able to split indole from the tryptophan molecule (*E. coli, Proteus vulgaris, Morganella and Edwardsiella*) from those that cannot (most *Shigella, Salmonella, Citrobacter, Klebsiella pneumoniae, Enterobacter, Serratia, Proteus mirabilis and Yersinia*). This test is also used to separate the Gram+ rods, *Bacillus alvei* (+) from *Bacillus* spp. (McFadden, p. 173).

FIGURE 27.1 Indole Test



CH_2CCOOH
H
NH_2
$+ H_2O$
tryptophanase cleaves indole from tryptophan
N
H
Tryptophan (in broth)
Indole
$+ CH_3CCOOH$
O
Pyruvate
$+ NH_3$
Ammonia
Addition of indicator, Kovac's reagent (*p*-dimethylaminobenzaldehyde HCl) produces the following reaction and color change:
Indole
+
CHO
$N(CH_3)_2$
Kovac's solution
C
$N(CH_3)_2$
Rosindole dye (Red ring formation)

(Organisms should be incubated at least 48 hours before addition of Kovac’s reagent.)

LABORATORY EXERCISE

MATERIALS

- ☑ 1 Tryptone broth or SIM medium (per organisms to be tested)
- ☑ Kovac’s reagent (*p*-dimethylaminobenzaldehyde-HCl in n-amyl alcohol)
- ☑ inoculating loop

PROCEDURE

1. Inoculate Tryptone Broth with organism. Incubate 18-24 hours.
2. Add 3 drops of Kovac's reagent.
3. Observe for color change.

Positive: red ring at top of broth
Negative: no color change

27.2 Methyl Red Test (Mixed Acid Fermentation).

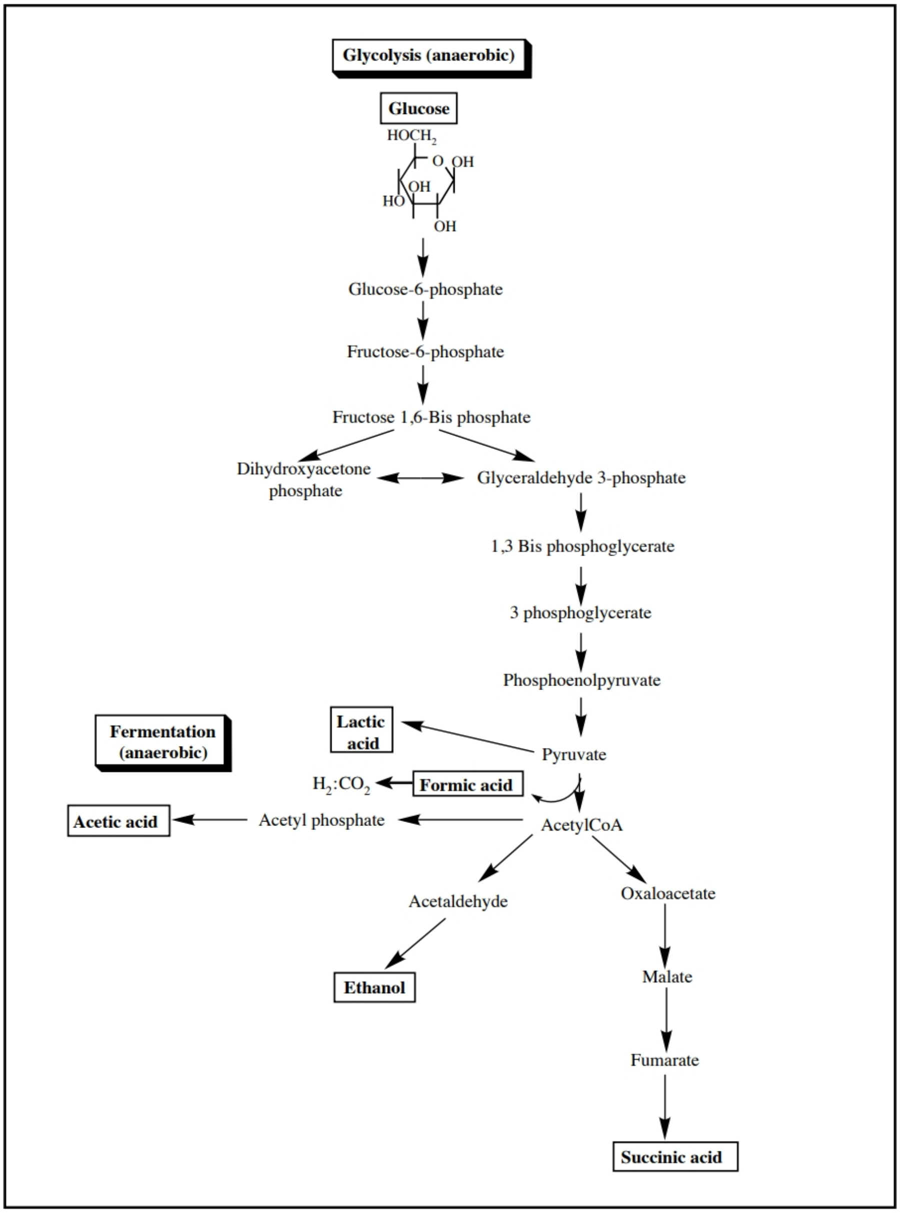
INTRODUCTION

The Methyl Red Test, or mixed acid fermentation, is one of the fermentative pathways found in the Enterobacteriaceae. The primary purpose of the Mixed Acid Fermentation is to differentiate between *E. coli* (+ MR) and *Enterobacter* (- MR). *E. coli* is a mixed acid fermenter, producing lactic, acetic, formic and succinic acids as well as ethanol in the process of fermenting glucose. The formic acid is immediately converted to H_2 and CO_2 (see Figure 27.2).

In order to obtain accurate results, the cultures of bacteria must be inoculated into MR-VP broth for a full 24 hour period at 30°C.

How the indicator, methyl red, works: the color change upon addition of methyl red to the MR-VP medium is simply an indication of a change in the pH of the solution. If the bacteria growing in the medium produce mixed acids in fermentation (lactic, acetic, formic and succinic acids) then the medium turns red. Methyl red turns red at pH 4-5 but turns yellow at pH 6-7.

FIGURE 27.2 Mixed acid fermentation (Methyl Red).



Glycolysis (anaerobic)
Glucose
HOCH2
O
OH
OH
HO
OH
Glucose-6-phosphate
Fructose-6-phosphate
Fructose 1,6-Bis phosphate
Dihydroxyacetone phosphate
Glyceraldehyde 3-phosphate
1,3 Bis phosphoglycerate
3 phosphoglycerate
Phosphoenolpyruvate
Fermentation (anaerobic)
Lactic acid
Pyruvate
H2:CO2
Formic acid
Acetic acid
Acetyl phosphate
AcetylCoA
Acetaldehyde
Oxaloacetate
Ethanol
Malate
Fumarate
Succinic acid

LABORATORY EXERCISE

SUPPLIES

- ☑ 1 MR-VP broth (per organism to be tested)
- ☑ Methyl red indicator
- ☑ inoculating loop

PROCEDURE

1. Inoculate the MR-VP broth with the organism. Incubate 48 hours.
2. Add 3–4 drops of methyl red indicator.
3. Observe color.

Positive: red color

Negative: yellow/orange color

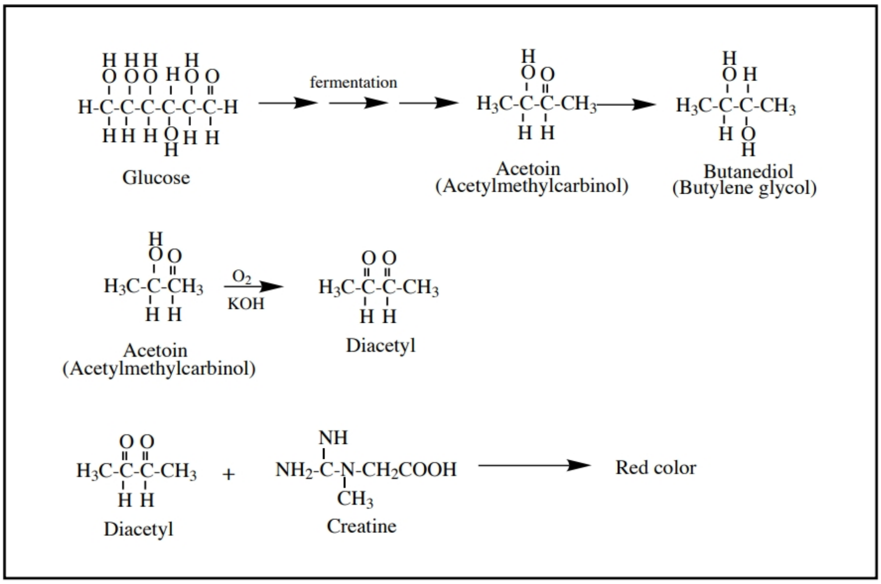
27.3 Voges-Proskauer Test (Butanediol Fermentation).

INTRODUCTION

Two pathways of glucose fermentation exist in the Enterobacteriaceae. Different members of this group may be able to do either (a) the mixed acid fermentation reactions (methyl red test) or (b) the butanediol fermentation (Voges Proskauer test) but not both reactions in the same organism at the incubation time used. Thus, the two fermentative pathways serve as differential tests for the Enterobacteriaceae.

In the following Voges-Proskauer reaction (Figure 27.3), glucose is fermented in a series of steps to acetylmethylcarbinol (acetoin) which is actually an intermediate in the reaction (Reaction 1). The acetoin intermediate, however, is important in the reaction mix with the indicators, α-naphthol and KOH (Reactions 2 and 3) with creatine (found in KOH mixture) which makes the mixture turn a red color (**positive reaction after 48 hours**).

FIGURE 27.3 Voges-Proskauer Test.


Glucose
fermentation
Acetoin
(Acetylmethylcarbinol)
Butanediol
(Butylene glycol)
O_2
KOH
Acetoin
(Acetylmethylcarbinol)
Diacetyl
Diacetyl
Creatine
Red color

(A stronger VP reaction may occur if tubes of 0.3-0.5 ml MR-VP broth are inoculated with organisms grown initially on MacConkey agar or TSI slants).

LABORATORY EXERCISE

MATERIALS

☑ 1 MR-VP broth (per organism to be tested)
☑ Barritt’s solution B (KOH)
☑ Barritt’s solution A (alpha-naphthol)
☑ inoculating loop

PROCEDURE

1. Inoculate the MR-VP broth with the organism. Incubate 48 hours.
2. Add 9 drops of Barritt’s solution A.
3. Add 9 drops of Barritt’s solution B.
4. Mix well. Let stand and watch for color change (will occur from 15 minutes to 2 hours).

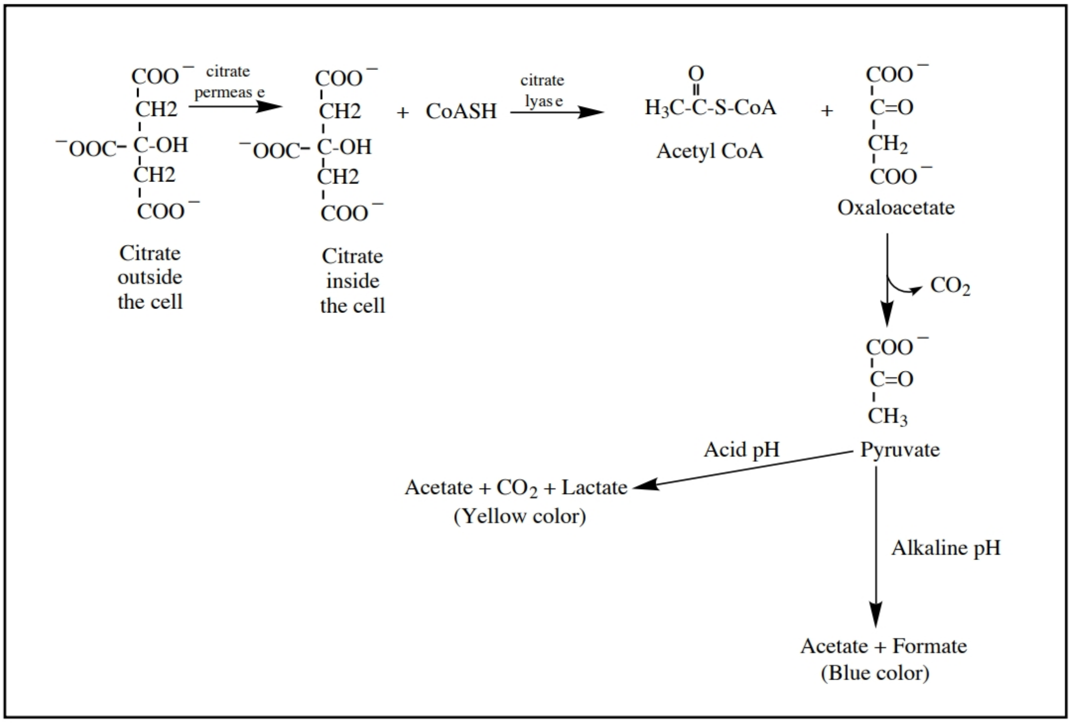
Positive: reddish to black color
Negative: no color change

27.4 Citrate Test.

INTRODUCTION

Citrate is an intermediate in the tricarboxylic acid cycle (Krebs cycle). Some bacteria are capable of taking citrate into the cell and using it as a carbon source. Other bacteria, such as *E. coli, Shigella, Yersinia* and *Morganella* cannot use citrate as a source of carbon as they produce no citrate permease and therefore are not capable of taking citrate into the cell. In organisms which can utilize citrate as the sole source of carbon (*Enterobacter, Salmonella, Serratia, Klebsiella)*, the reactions seen in Figure 27.4 occur.

FIGURE 27.4 Citrate Test.



citrate permease
citrate lyase
Citrate outside the cell
Citrate inside the cell
+ CoASH
Acetyl CoA
Oxaloacetate
CO2
Pyruvate
Acid pH
Acetate + CO2 + Lactate (Yellow color)
Alkaline pH
Acetate + Formate (Blue color)

(The test is also used to differentiate *Burkholderia cepacia* (+) from *Stenotrophomonas maltophilia* (-)).

LABORATORY EXERCISE

MATERIALS

☑ 1 Citrate slant (per organism to be tested)

☑ inoculating loop

PROCEDURE

1. Inoculate citrate slant (contains bromothymol blue indicator) and incubate 18-24 hours (lid should not be tight).
2. Observe for color change.

Positive: change to blue

Negative: remains green

Laboratory Report at end of Laboratory 30.

LABORATORY TWENTY-EIGHT

Sugar Fermentations

LEARNING OBJECTIVE

Students will be able to:

- Identify which organisms ferment glucose to produce an acid by product and which organisms produce gas from glucose fermentation

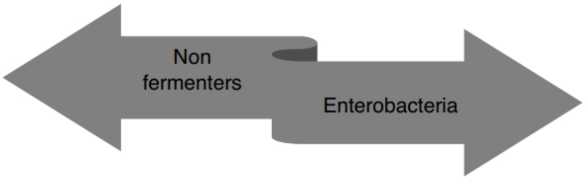
PURPOSE

To identify the products of bacterial fermentation of sugars.

INTRODUCTION

Glucose fermentation: The fermentation of glucose, $C_6H_{12}O_6$, is an anaerobic process in which the glucose is broken down to acids and other gaseous byproducts (H_2 and CO_2). The formation of acids on the growth medium causes the pH indicator, phenol red, to change from a red color to yellow. The entrapment of gas bubbles in the inverted Durham tube inserted in the broth tube is an indication that gases have been produced. (See Mixed Acid Fermentation in Exercise 27 for additional explanation.)

We will be able to differentiate between glucose fermenters (e.g. members of the Enterobacteriaceae) and the non-glucose fermenters such as *Pseudomonas aeruginosa (-)*.



LABORATORY EXERCISE

Each student will select one or more of the following organisms and perform each test.

ORGANISMS FOR GLUCOSE FERMENTATION

☑ *Citrobacter freundii*
☑ *Enterobacter aerogenes*
☑ *Escherichia coli*
☑ *Klebsiella pneumoniae*
☑ *Proteus vulgaris*
☑ *Pseudomonas aeruginosa*
☑ Other Enterobacteria

28.1 Glucose Fermentation.

SUPPLIES

☑ 1 Glucose broth with Durham tube (per organism to be tested)
☑ inoculating loop

PROCEDURE

1. Inoculate Glucose broth (contains phenol red indicator) which has Durham tube. Incubate 18-24 hours.
2. Observe for color change and presence of gas bubble in Durham tube.

Positive: (acid) yellow color, (gas) bubble in Durham tube

Negative: (no acid) red color, (no gas) no bubble

Laboratory Report at end of Laboratory 30.

LABORATORY TWENTY NINE

Motility

LEARNING OBJECTIVES

Students will be able to:

- Describe bacterial motility in a drop of water
- Interpret the results of the combined biochemical tests for sulfide production, indole oxidation and motility.

PURPOSE

To observe bacterial motility.

INTRODUCTION

Bacterial movement may be studied by making a conventional wet mount (placing a drop of water on a slide and placing a cover slip atop the drop of water). Since the activity of the microorganisms may be somewhat inhibited by the constraints of the coverslip and slide, the hanging drop method may be used instead. Using this method, a drop of bacterial growth medium containing microorganisms is suspended from the bottom of a cover slip within an indentation (a well) on a special slide. The organisms are then free to move about as they would in nature.

Bacterial motility is determined by types and numbers of flagella contained on the single-celled organisms. Some cyanobacteria move by gliding but most bacteria that move do so because of flagellar movement. Eukaryotic microorganisms may possess cilia or a different type of flagellum than that of a bacterium.

When the results of three tests may be seen with the inoculation of one tube, it saves time and money. The SIM (Sulfide-Indole-Motility) Test is such an example. One of the tests in the SIM test, the **Indole test,** has been explained previously in the IMVC set of test (see Exercise 27.1)

The **Sulfide test** is used to detect the ability of an organism to produce H_2S from the reduction of sulfur in the SIM medium to sulfide.

The Motility test is an indication of an organism's ability to move away from the inoculation site (usually a stab into soft agar).

LABORATORY EXERCISE

Each student will select one or more of the following organisms and perform each test.

ORGANISMS

- ☑ *Escherichia coli*
- ☑ *Citrobacter freundii*
- ☑ *Morganella morganii*
- ☑ *Edwardsiella tarda*
- ☑ *Providencia stuartii*
- ☑ *Serratia marcescens*
- ☑ *Pseudomonas fluorescens* or *Pseudomonas putida*
- ☑ *Acinetobacter bayly*
- ☑ Other Enterobacteria

29.1 Hanging Drop Slide.

MATERIALS

- ☑ Hanging drop slide
- ☑ cover slips
- ☑ Vaseline petroleum jelly
- ☑ toothpicks
- ☑ inoculating loop

PROCEDURE

1. Place a cover slip on the table. Add a small amount of Vaseline to each corner using a toothpick.
2. Transfer 1-2 loopfuls of organism to the center of the cover slip to make a small drop.
3. Invert a hanging drop slide and carefully place onto the cover slip with the culture drop positioned in the center of the depression. Press gently to make contact with the Vaseline. Lift and invert in one quick motion.
4. Place on microscope and observe.

29.2 Motility Test Medium.

MATERIALS

☑ 1 tube of Motility Test medium (or SIM) (per organism to be tested)

☑ inoculating needle

PROCEDURE

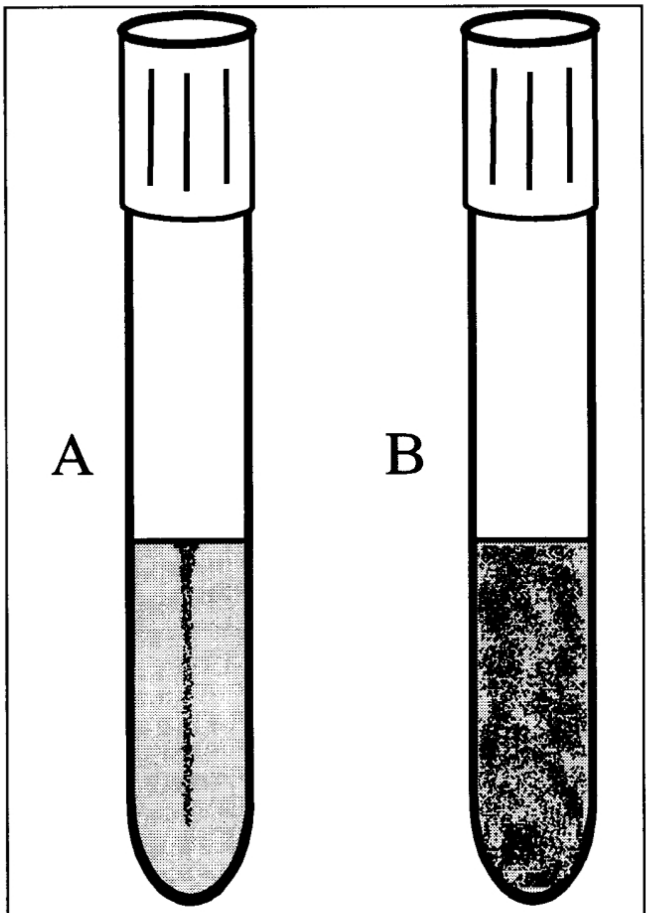
1. Inoculate using an inoculating needle to make a single stab (see Exercise 14.1). Incubate.
2. Observe growth in tube.

Positive: growth outward from stab, throughout medium

Negative: growth only at the stab site

Laboratory Report at end of Laboratory 30.

FIGURE 29.1 Patterns of growth in motility test medium.



Pattern of growth in motility test medium for: A. non-motile organism, and B. motile organism.

LABORATORY THIRTY

Other Biochemical Tests

30.1 Starch Hydrolysis.

LEARNING OBJECTIVE

Students will be able to:

- Describe the purpose, indicators, and interpretation of results for the starch hydrolysis and urease tests.

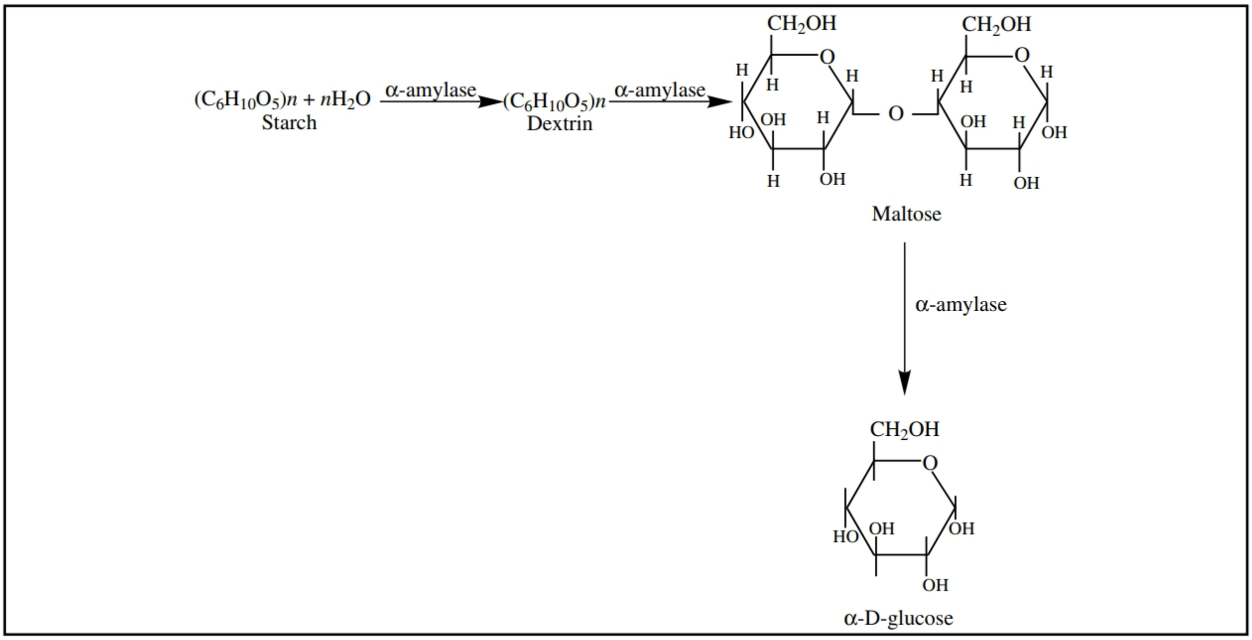
PURPOSE

To determine which organisms contain enzymes (amylases) to break down starch to other sugars.

INTRODUCTION

Starch is a complex carbohydrate produced by plants. Just as in the simple test of placing iodine on a potato, thus turning the starch-rich potato black, a similar test for starch consumption by bacteria is utilized in microbiology laboratories. Some types of soil bacteria produce **amylases**, enzymes which break down amylose and amylopectin to maltose, glucose and

FIGURE 30.1 Starch Hydrolysis.


$(C_6H_{10}O_5)n + nH_2O$
Starch
α-amylase
$(C_6H_{10}O_5)n$
Dextrin
α-amylase
CH_2OH
Maltose
α-amylase
CH_2OH
α-D-glucose

dextrins. In order to detect the organisms' ability to metabolize starch on an agar plate with starch added as a carbon source, an indicator such as iodine is used. In the areas where the bacteria have "eaten" the starch, there will be a clear area that contains no more starch; therefore, the iodine will not turn that area black (since it contains no more starch), but in the areas on the plate where the starch has not been metabolized the iodine will turn it black.

The reaction in Figure 30.1 shows how the reaction is carried out by *Bacillus.*

LABORATORY EXERCISE

ORGANISMS

(liquid culture in tube)

☑ *Bacillus megaterium*

☑ *Staphylococcus* spp

MATERIALS

☑ 1 Starch agar plate

☑ Gram's iodine

☑ inoculating loop

PROCEDURE

1. Divide plate in two halves by drawing line on the bottom.
2. Inoculate one half with a single streak of *Bacillus megaterium.*
3. Inoculate the other half in the same manner with a single streak of *Staphylococcus* spp.
4. Incubate for 24 hours.
5. Cover growth with several drops of Gram's iodine. Note color changes.

Positive: clear zone visible around growth

Negative: entire area around growth turns dark

30.2 Urease.

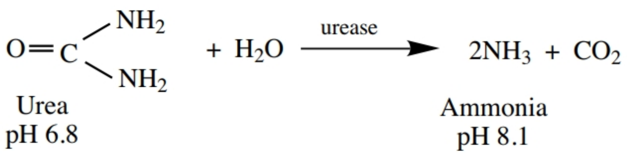
PURPOSE

To determine if an organism contains the urease enzyme and thus can metabolize urea.

INTRODUCTION

Proteus is a Gram^{-}rod which produces the urease enzyme to break down urea to carbon dioxide, water and ammonia. This test is used to differentiate the *Proteus* group of bacteria from other Gram negative pathogens such as *E. coli. Proteus* is an opportunistic pathogen responsible for infections in the urinary tract.

FIGURE 30.2 Urease Test



This reaction (Fig. 30.2) is an indication of how the urease enzyme works.

The indicator in this reaction is phenol red which gives an indication that the pH has changed to a more alkaline state. At pH 6.8 phenol red is an orange color; at pH 8.1 the urease reagent turns bright pink (fuschia).

A *false-positive* may occur when such organisms as *Pseudomonas* spp are grown in urease broth. *Pseudomonas* breaks down peptones in the broth thus making it more alkaline and inducing a color change to pink as if the urease were present when in fact *it is not.*

LABORATORY EXERCISE

Each student will select one or more of the following organisms and perform the test.

ORGANISMS

☑ *Escherichia coli*
☑ *Citrobacter freundii*
☑ *Morganella morganii*
☑ *Edwardsiella tarda*
☑ *Providencia stuartii*
☑ *Serratia marcescens*
☑ *Pseudomonas fluorescens*
☑ Other Enterobacteria

MATERIALS

☑ 1 Urea broth (per organism to be tested)
☑ inoculating loop

PROCEDURE

1. Inoculate urea broth (contains phenol red) with organism. Incubate for 18-24 hours.
2. Observe color change.

Positive: Bright pink/red color

Negative: No color change

BIOCHEMICAL TEST INTERPRETATION CHART

GRAM-NEGATIVE BACTERIA

Test	**Indicator**	**Reagents**	**Test for**	**Negative**	**Positive**
Glucose					
Lactose					
Methyl Red (MR-VP broth)					
Vogues Proskauer (MR-VP broth)					
Sulfide (SIM)					
Indole (SIM)					
Motility (SIM)					
Urea					
Citrate					
Oxidase					
GRAM-POSITIVE BACTERIA					
Starch					
Catalase					
MSA					

LABORATORY REPORT 27-30
BIOCHEMICAL TESTS

Name:____

RESULTS

Organims	Glucose	Lactose	Gas (glue. or lactose)	Indol	Motility	Citrate	Urea	H_2S	MR	VP	Oxidase	Catalase	MSA	Starch
Ecoli												NA	NA	NA
Citrobacter freundii												NA	NA	NA
Morganella morganii												NA	NA	NA
Acinetobacter bayly												NA	NA	NA
Serratia marcescens												NA	NA	NA
Enterobacter aerogenes												NA	NA	NA
Pseudomonas fluorescens or *P.putida*												NA	NA	NA
S.epidermids **G+cocci**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA			
S.saprophyticus **G+cocci**	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA			
E.raffinosus **G+cocci**	N/A	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA		NA	
B.megaterium **G+ rods**	N/A	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA		NA	

LABORATORY THIRTY-ONE

Unknown Identification

LEARNING OBJECTIVES

Students will be able to:

- Successfully isolate a bacterial unknown from a mixed culture
- Identify a bacterial unknown using biochemical test and staining methods learned in this course

PURPOSE

The purpose of this experiment is to use the techniques learned in the laboratory to identify unknown bacteria.

INTRODUCTION

Bacterial cultures in nature are generally mixed cultures. Whether from soil samples or clinical specimens, the task of isolating and identifying the different bacteria within these mixtures is the job of the microbiologist.

Throughout the laboratory exercises, many techniques have been utilized to learn about the characteristics of various bacteria. In this exercise, aseptic techniques, staining procedures, and biochemical tests will all be performed in the identification of unknown bacteria.

The identification of unknowns is more than just a series of procedures. Careful planning and observation are important in order to isolate and characterize the bacteria in a quick and efficient manner. For example, Gram staining of the culture upon receipt gives information on the morphology of the organisms, while also providing hints on which biochemical tests may later be employed. Diligent notation of characteristics observed at each step of the investigation will provide all the information necessary to determine the next tests to be performed, as well as help support the eventual identification.

Each student will receive a culture tube containing a mixture with one Gram-positive and one Gram-negative organism. Follow the outline provided in the procedures to isolate and identify each.

LABORATORY EXERCISE

31.1 Unknown Identification.

ORGANISMS

(liquid culture in tube)

Gram-positive:

☑ *Bacillus megaterium*
☑ *Enterococcus faecium*
☑ *Staphylococcus saprophyticus*
☑ *Staphylococcus epidermidis*

Gram-negative:

☑ *Escherichia coli*
☑ *Citrobacter freundii*
☑ *Morganella morganii*
☑ *Edwardsiella tarda*
☑ *Providencia stuartii*
☑ *Serratia marcescens*
☑ Other Enterobacteria
☑ *Pseudomonas fluorescens*

MATERIALS

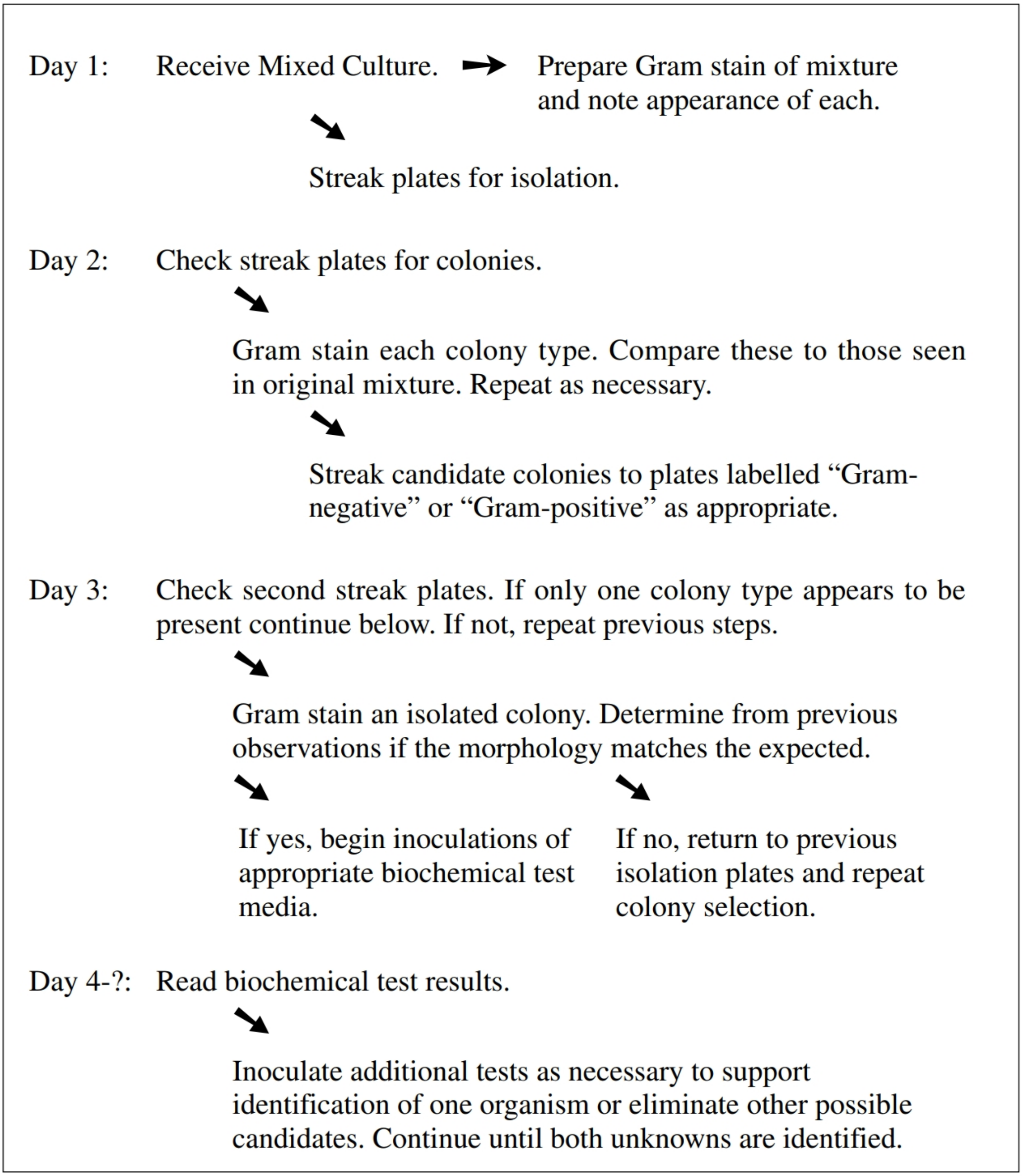
☑ Nutrient agar plates
☑ Nutrient broth tubes
☑ Gram stain reagents
☑ Biochemical test reagents and media
☑ slides
☑ inoculating loop
☑ inoculating needle

PROCEDURE

1. Obtain an unknown mixture from the TA. Immediately write down the unknown number.
2. Streak for isolation on two nutrient agar plates. These are the primary isolation plates. Incubate.
3. Make a Gram stain of the mixed culture. Observe under oil immersion and identify the cell morphology of each type of bacteria in the mixture. Note which is Gram-positive and which is Gram-negative.
4. Check the primary isolation plates after 18-24 hours incubation. Note size and appearance of isolated colonies. From each type, select a well-isolated colony and carefully remove a small portion for a Gram stain. Compare the observed cells to those from the mixed culture Gram stain. Once the two cell types have been identified, make two streak plates for isolation from each using the same colonies as for the Gram stains. Label all plates well before incubating.
5. After 18 - 24 hours, check the streak plates. Repeat step 4 until pure cultures are obtained for both bacterial types in the original mixture. Show the plates containing the pure cultures and the Gram stains from each to the TA.

6. Inoculate appropriate biochemical tests from the pure cultures. Follow a logical series of tests to narrow down the possible organisms until all but one Gram-positive and one Gram-negative have been eliminated.
7. Detail the findings on the laboratory report.

FIGURE 31.1 Flow chart for unknown identification.



Day 1: Receive Mixed Culture. → Prepare Gram stain of mixture and note appearance of each.

↘ Streak plates for isolation.

Day 2: Check streak plates for colonies.

↘ Gram stain each colony type. Compare these to those seen in original mixture. Repeat as necessary.

↘ Streak candidate colonies to plates labelled "Gram-negative" or "Gram-positive" as appropriate.

Day 3: Check second streak plates. If only one colony type appears to be present continue below. If not, repeat previous steps.

↘ Gram stain an isolated colony. Determine from previous observations if the morphology matches the expected.

↘ If yes, begin inoculations of appropriate biochemical test media.

↘ If no, return to previous isolation plates and repeat colony selection.

Day 4-?: Read biochemical test results.

↘ Inoculate additional tests as necessary to support identification of one organism or eliminate other possible candidates. Continue until both unknowns are identified.

LABORATORY REPORT 31
UNKNOWN IDENTIFICATION

Name:______________________________________

RESULTS

Unknown #: ________

TA Approvals:

Mixed culture Gram stain: _______

Gram-positive organism: Pure plate ________ Gram-stain ________

Gram-negative organism: Pure plate ________ Gram-stain ________

Biochemical tests performed and results:

CONCLUSIONS

Write a short paragraph for each of the two unknowns describing what each is and how it was determined.

SECTION NINE

APPENDICES

APPENDIX A

Media and Biochemical Formulations

GROWTH MEDIA

Many of these ingredients and pre-mixed media formulations are available from Difco Laboratories (Detroit, MI).

AMES TOP AGAR

Prepare top agar base containing 0.5 g sodium chloride and 0.65 g agar in distilled water to 100 ml total volume. Autoclave. Cool and store with lid tightened. Prepare Histidine/Biotin stock solution containing 12.37 mg D-Biotin (F.W. 247.3) and 9.6 mg L-Histidine in distilled water to 100 ml total volume. Filter sterilize. Just prior to use, liquify 100 ml of the agar base and cool to 45°. Add 10 ml of the Histidine/Biotin stock solution and mix well. Aseptically distribute 2 ml each into sterile test tubes. Keep liquified at 45°C until use.

CITRATE AGAR (SIMMON'S)

Per liter:

Magnesium sulfate	0.2 g
Ammonium dihydrogen phosphate	1 g
Dipotassium phosphate	1 g
Sodium citrate	2 g
Sodium chloride	5 g
Agar	15 g
Bromthymol blue	0.08 g

pH 6.8

DECA-STRENGTH PHAGE BROTH (DSPB)

Per liter:

Peptone	100 g
Yeast extract	50 g
Sodium chloride	25 g
Dibasic potassium phosphate	80 g

pH 7.6

EOSIN-METHYLENE BLUE (EMB) AGAR

Per liter:

Peptone	10 g
Dipotassium phosphate	2 g
Agar	15 g
Eosin Y	0.4 g
Methylene blue	0.065 g

After autoclaving, add sterile lactose to 1% concentration to test lactose fermentation.

FLUID THIOGLYCOLLATE MEDIUM

Per liter:

Yeast extract	5 g
Casitone	15 g
Dextrose	5.5 g
Sodium chloride	2.5 g
L-Cystine	0.5 g
Sodium thioglycollate	0.5 g
Agar	0.75 g
Resazurin	0.001 g

GLUCOSE MINIMAL AGAR

1. Prepare 10X PN solution (per liter):

Monobasic potassium phosphate	91 g
Sodium hydroxide	16.8 g
Ammonium sulfate	12 g

2. Prepare 200X R-salts solution:

10% Magnesium sulfate	400 ml
1% Ferrous sulfate	100 ml
Hydrochloric acid (concentrated)	2 ml

3. In distilled water, dilute PN solution and R-salts to 1X concentrations, and add glucose to 20 mM final concentration.

GLUCOSE TEST BROTH (PHENOL RED BROTH BASE)

Per liter:

Proteose peptone No. 3	10 g
Beef extract	1 g
Sodium chloride	5 g
Phenol red	0.018 g

pH 7.4. After autoclaving, aseptically add sterile glucose to 1% final concentration.

HEKTOEN-ENTERIC (HE) AGAR

Per liter:

Proteose Peptone, Difco	12 g
Yeast Extract	3 g
Bile salts No. 3	9 g
Lactose	12 g
Saccharose	12 g
Salicin	2 g
Sodium chloride	5 g
Sodium thiosulfate	5 g
Ferric ammonium ctirate	5 g
Agar	14 g
Bromthymol blue	0.065 g
Acid fuchsin	0.1 g

LACTOSE BROTH WITH BROMOTHYMOL BLUE INDICATOR

Per liter:

Beef extract	3 g
Peptone	5 g
Lactose	5 g
Bromthymol blue	0.06 g

pH 6.9

LITMUS MILK MEDIUM

Per liter:

Skim milk	100 g
Litmus	5 g

pH 6.8

MACCONKEY AGAR

Per liter:

Peptone	17 g
Proteose peptone, Difco	3 g
Lactose	10 g
Bile salts No. 3	1.5 g
Sodium chloride	5 g
Agar	13.5 g
Neutral red	0.03 g
Crystal violet	0.001 g

pH 7.1

MANNITOL SALT AGAR (MSA)

Per liter:

Proteose peptone No. 3, Difco	10 g
Beef extract	1 g
D-Mannitol	10 g
Sodium chloride	75 g
Agar	15 g
Phenol red	0.025 g

pH 7.4

MOTILITY TEST MEDIUM

Per liter:

Tryptose	10 g
Sodium chloride	5 g
Agar	5 g

To assist in visualizing growth, the indicator triphenyltetrazolium can be added to the media (to 0.005% final concentration) prior to autoclaving.

MR-VP BROTH

Per liter:

Buffered peptone	7 g
Dipotassium phosphate	5 g
Dextrose	5 g

pH 6.9

MUELLER-HINTON AGAR

Per liter:

Beef, Infusion from	300 g
Casamino acids, technical	17.5 g
Starch	1.5 g
Agar	17 g

pH 7.3

NUTRIENT AGAR

Per liter:

Beef extract	3 g
Peptone	5 g
Agar	15 g

pH 6.0

NUTRIENT BROTH

Per liter:

Beef extract	3 g
Peptone	5 g

pH 6.8

p-HYDROXYBENZOATE (POB) AGAR

(See Glucose minimal medium for PN and R-salt stocks)

PN solution	1X
R-salts solution	1X
p-hydroxybenzoate	5 mM final

SABOURAUD DEXTROSE AGAR

Per liter:

Neopeptone, Difco	10 g
Dextrose	40 g
Agar	15 g

pH 5.6

SIM MEDIUM

Per liter:

Peptone	30 g
Beef extract	3 g
Peptonized iron, Difco	0.2 g
Sodium thiosulfate	0.025 g
Agar	3 g

pH 7.3

STARCH AGAR

Per liter:

Beef extract	3 g
Soluble starch, Difco	10 g
Agar	12 g

pH 7.5

SUCROSE BROTH WITH BROMOTHYMOL BLUE INDICATOR

Per liter:

Beef extract	3 g
Peptone	5 g
Sucrose	5 g
Bromthymol blue	0.06 g

pH 6.9

m-TOLUATE MINIMAL AGAR

(See Glucose minimal medium for PN and R-salt stocks)

PN solution	1X
R-salts solution	1X
m-toluate	mM final

For plates requiring leucine, add leucine to 25 μg/ml final concentration.

TRIPLE SUGAR IRON (TSI) MEDIUM

Per liter:

Beef extract	3 g
Yeast extract	3 g
Peptone	15 g
Proteose peptone, Difco	5 g
Dextrose	1 g
Lactose	10 g
Sucrose	10 g
Ferrous sulfate	0.2 g
Sodium chloride	5 g
Sodium thiosulfate	0.3 g
Agar	12 g
Phenol red	0.024 g

pH 7.4

TRYPTIC SOY AGAR (TSA)

Per liter:

Tryptone	15 g
Soytone	5 g
Sodium chloride	5 g
Agar	15 g

pH 7.3

TRYPTIC SOY BROTH (TSB)

Per liter:

Tryptone	15 g
Soytone	5 g
Sodium chloride	5 g

pH 7.3

TRYPTONE AGAR

Per liter:

Tryptone	10 g
Agar	15 g

TRYPTONE BROTH

Per liter:

Tryptone	10 g

TRYPTONE SOFT AGAR

Per liter:

Tryptone	10 g
Agar	4 g

UREA BROTH

Per liter:

Yeast extract	0.1 g
Potassium phosphate, monobasic	9.1 g
Potassium phosphate, dibasic	9.5 g
Urea, Difco	20 g
Phenol red	0.01 g

pH 6.8

BIOCHEMICAL FORMULATIONS

Acid-alcohol

95% ethanol	97 ml
Hydrochloric acid (concentrate)	3 ml

1% Agarose in TBE

Add 1 g of agarose to a total volume of 100 ml of TBE buffer in a flask. Weigh. Heat to boiling. Cool briefly and reweigh, adding distilled water to return to previous weight. When warm to the touch, pour into gel mold.

Barritt's solution A (alpha-naphthol)

Alpha-naphthol	5 g
95% ethanol	100 ml

Barritt's solution B (KOH)

Potassium hydroxide	40 g
Creatine	0.3 g
distilled water	100 ml

Carbolfuchsin

Prepare 0.3 g of basic fuchsin in 10 ml of 95% ethanol. Mix 5 g phenol with 95 ml of distilled water. Combine the two solutions and let stand for several days. Filter through paper and store until use.

20% Copper sulfate

Dissolve 20 g of copper sulfate in distilled water to 100 ml total volume.

Crystal violet

Prepare 2 g of crystal violet in 20 ml of 95% ethanol. Mix 0.8 g of ammonium oxalate in 80 ml distilled water. Combine the two solutions and store for 24 hours. Filter through paper and store until use.

70% Ethanol

Absolute ethanol	70 ml
distilled water	30 ml

Ethidium bromide (EtBr) gel staining solution

Ethidium bromide	0.4 mg
distilled water	100 ml

Gram's iodine

Dissolve 2 g of potassium iodide in 300 ml of distilled water. When dissolved, add 2 g of iodine crystals.

3% Hydrogen peroxide

30% Hydrogen peroxide	10 ml
distilled water	90 ml

Kovac's reagent

n-amyl alcohol	75 ml
Hydrochloric acid (concentrated)	25 ml
p-dimethyl-aminobenzaldehyde	5 g

Dissolve reagent completely in n-amyl alcohol at 37°C, then carefully add hydrochloric acid while stirring.

Linder's cotton blue

Phenol crystals	20 g
Lactic acid	20 ml
Glycerol	40 ml
Cotton blue	0.05 g

Dissolve the phenol crystals in the other ingredients by heating the mixture gently under hot water.

Malachite green

Malachite green oxalate	0.5 g
distilled water	100 ml

Methyl red

Methyl red	0.2 g
95% ethanol	500 ml
distilled water	500 ml

Methylene blue (Loeffler's)

Dissolve 0.3 g of methylene blue in 30 ml of 95% ethanol. Mix 0.01 potassium hydroxide in 100 ml of distilled water. Combine the two solutions.

Nigrosin

Nigrosin	10 g
distilled water	100 ml

Boil for 30 minutes. Filter twice through double filter paper and store aseptically.

4-nitro-o-phenylenediamine (4-NOPD) solution

Caution: mutagen

4-NOPD	100 μg
distilled water	10 ml

Oxidase reagent

Dimethyl-p-phenylenediamine-HCL	1 g
distilled water	100 ml

Make fresh daily and store in refrigerator.

Safranin

Safranin O	0.25 g
95% ethanol	10 ml

Dissolve in ethanol. Add to 100 ml of distilled water.

Sodium acetate, 3 M, pH 5.2

Dissolve 408 g sodium acetate$\cdot 3H_2O$ in water. Adjust pH to 5.2 with 3 M acetic acid. Add water to one liter.

TENS solution

Prepare fresh just prior to use:

TE buffer	3.875 ml
0.5 M Sodium hydroxide	1 ml
20% Sodium dodecylsulfate (SDS)	0.125 ml

Tris-Boric Acid-EDTA (TBE) buffer, ph 8.0

Tris base	89 mM
Boric acid	89 mM
Ethylenediamine tetraacetic acid (EDTA)	2 mM

Tris-EDTA (TE) buffer

Tris-HCl, pH 8.0	10 mM
EDTA, pH 8.0	1 mM

APPENDIX B

References and Suggested Readings

REFERENCES

Atlas, R.M. *Principles of Microbiology.* (1997) W.C. Brown, Dubuque, IA.

Benson, H.J. *Microbiological Applications Laboratory Manual in General Microbiology.* (1998) WCB/McGraw-Hill, Boston, MA.

Blazevic, D.J. & Ederer, G.M. *Principles of Biochemical Tests in Diagnostic Microbiology.* (1975) John Wiley & Sons, New York, NY.

Crabtree, K.T. & Hinsdill, R.D. VFundamental Experiments in Microbiology. (1974) W.B. Saunders Co., Philadelphia, PA.

Glick, B.R. & Pasternak, J.J. *Molecular Biotechnology Principles & Applications of Recombinant DNA*. (1994) ASM Press, Washington, D.C.

Hughes, M.N. & Poole, R.K. Metal mimicry and metal limitation in studies of metal-microbe interactions. in *Metal-Microbe Interactions*. (1989) ed. Poole, R.K. & Gadd, G.M. IRL Press, Oxford University Press, Oxford, England.

Ingraham, J.L. & Ingraham, C.A. *Introduction to Microbiology*. (1995) Wadsworth Publishing Co., Belmont, CA.

Leboffe, M.J. & Pierce, B.E. *A Photographic Atlas for the Microbiology Laboratory.* (1996) Morton Publishing Co. Englewood, CO.

Neidhardt, F.C., Ingraham, J.L., Schaechter, M., *Physiology of the Bacterial Cell: A Molecular Approach*. (1990) Sinauer Associates, Sunderland, MA.

Pelczar,Jr., M.J. and Chan, E.C.S. *Laboratory Exercises in Microbiology*. (1977) McGraw-Hill, Inc. New York, NY.

Pelczar, Jr., M.J., Reid, R.D. and Chan, E.C.S. *Microbiology*. (1977) McGraw-Hill, Inc. New York, NY.

Prescott, L.M., Harley, J.P., Klein, D.A., *Microbiology.* (1996) Wm. C. Brown Publishers, Dubuque, IA.

Perry, J.J. & Staley, J.T. *Microbiology Dynamics and Diversity*. (1997) Saunders College Publishing, Orlando, FL.

Smoot, R.C., Price, J. *Chemistry A Modern Course*. (1975) Charles E. Merrill Publishing Co., Columbus, Ohio.

Stanier, R.Y., Ingraham, J.L., Wheelis, M.L. & Painter, P.R. *The Microbial World*. (1986) Prentice-Hall, Englewood Cliffs, NJ.

Stryer, L. *Biochemistry*. W.H. Freeman and Company, New York. 1995.